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Cryopreservation of Gametes and Larvae of the Eastern Oyster Crassostrea Virginica.

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CRYOPRESERVATION OF GAMETES AND LARVAE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

A Dissertation

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To my son

Let us royster with the oyster-

in the shorter days and moister,

They are brought by brown September,

with its roguish final R;

For breakfast or for supper,

on the under shell or upper,

Of dishes he's the daisy,

and of shell-fish he's the star.

So welcome with September

to the knife and glowing ember,

Juicy darling of our dainties,

dispossessor of the clam!

Detroit Free Press, 1889

"Oysters possess, when young, the faculty of swimming by means of a simple yet admirable development of their powers; but, when arrived at full growth, this faculty or inclination ceases, and, while some of their active relatives are darting round them, they remain contentedly in their place of abode, surrounded by a numerous and continually increasing progeny"

Mary Roberts: A Popular History of the Mullusca, 1851.

"Oysters have more to worry about than we have and less to worry with"

Anonymous

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ABSTRACT

Storage of gametes and larvae offers benefits for research and commercial oyster production and should be applicable in the hatchery at a commercial scale. We optimized refrigerated storage of eastern oyster sperm. Significant differences were found in the motility of sperm suspended in artificial sea water (ASW) of different osmotic pressures ($P < 0.001$). The highest motility was found in undiluted sperm and the lowest in 1:31 dilution. The greatest larval survival at 12 h (48%) was obtained with sperm diluted in calcium-free Hanks' balanced salt solution (C-F HBSS). Thus, for storage of longer than 1 d, it is best to leave samples undiluted. However, when sperm samples are diluted, it is best to maintain high sperm concentrations and to use C-F HBSS as an extender.

Samples were frozen at –2.5°C/min, held for 5 min at –30°C, and plunged in liquid nitrogen using 10% propylene glycol (PG) as the cryoprotectant for sperm and 10% or 15% PG for trochophore larvae. Motility and fertilizing ability of thawed sperm were affected by cryoprotectant concentration and thawing temperature ($P = 0.0001$). Larval survival was affected by the concentration of larvae per straw ($P = 0.001$).

Frozen samples were transported to an oyster hatchery at Grand Isle, Louisiana. After 4 months, 1,000 oysters from the control group, 230 oysters produced from thawed sperm, and 850 oysters from thawed larvae were found. Oysters produced from thawed larvae developed normally in the hatchery, demonstrating opportunities for use of cryopreservation in research and aquaculture.

Flow cytometry with the fluorescent dyes Sybr-14 and propidium iodide (PI) was used to assess membrane damage of thawed sperm, and rhodamine 123 and PI were used to assess mitochondrial function. Preliminary studies of cryopreservation of oyster eggs

were performed. Fluorescein diacetate (FDA) was used to identify viable eggs. Dimethyl sulfoxide (0.88 M and 1.75 M) and sucrose (0.12 M and 0.25 M) were the least toxic cryoprotectants evaluated. The cooling rate yielding least damage to eggs was -1.5°C per min. However, only an average of 14 eggs (out of 200) were stained with FDA in thawed samples and none were fertilizable.

CHAPTER 1
FOREWORD

The most important bivalve species in the United States is the eastern oyster, Crassostrea virginica (Wendell and Malone 1994). However, along the Atlantic and Gulf coasts, oyster production had declined due to reasons including a lack of consistent seed supply, excessive harvest, disease and natural predation (Supan and Wilson 1993). The use of cryopreserved gametes and larvae could improve hatchery production of seedstock to increase production for the Louisiana oyster industry. Cryopreservation of oyster gametes and larvae has been tested only at the laboratory level, but given the benefit that this technique offers, cryopreservation of oyster gametes and larvae should be developed for commercial application at the hatchery level.

Refrigerated storage of fish sperm is well studied (Stoss et al. 1978, Hara et al. 1982), although studies of refrigerated storage of oyster sperm are lacking. Short-term storage of oyster sperm (for a few hours or days) can be useful for genetic studies and artificial breeding programs. Short-term storage offers the possibility of controlling factors that can affect sperm quality such as osmotic pressure and bacterial contamination. Another benefit of short-term storage and dilution of oyster sperm is use for refinement of cryopreservation techniques.

Gametes can be cryopreserved for several reasons: (1) to facilitate the management of seed production; (2) to supply research material; (3) to preserve genetic diversity, and 4) to select and preserve genes of individuals with desirable characteristics for future development. Techniques for cryopreservation of ova, sperm and embryos of mammals such as boar (Bwanga 1991), stallion (Amann and Pickett 1987), mouse (Centola et al. 1992) and man (Van Blerkom and Davis 1994) have been developed.

Within aquatic species most work on cryopreservation has addressed sperm, with some 200 species of fish studied (Stoss 1983, Rana 1996). Cryopreservation of gametes is poised to assume a role in seed production and genetic management of broodstock; however, research has largely been confined in aquaculture to spermatozoa of three groups of commercially important fishes: salmonids, tilapias and carps (Stoss 1983).

Despite the advantages of gamete cryopreservation, work focusing on molluskan gametes is scarce (Gwo 1995). For mollusks, germplasm repositories where gametes and larvae can be stored frozen for long periods could play a crucial role in genetic management by preserving desirable lines and making genetic and breeding studies possible. The year-round availability of top-quality bivalve larvae is a major problem in the field of mollusk farming (Micarelli 1994) and cryopreservation is an option to resolve this problem. Farmers could use facilities for seed production regardless of the spawning season (Gwo 1995), storage of larvae could be a useful tool in the management of bivalve stocks (Renard 1991), frozen eggs and larvae could provide feed for larval marine fish and genetically modified stocks could be made available. These points are addressed in Chapter 2.

Several problems exist with techniques of cryopreservation. First, cryopreservation methods developed for one species are not always applicable to other species. Second, reproducible techniques for long-term storage need to be refined. Third, the problems associated with the cryopreservation of eggs and larvae are more complex than those of sperm. These problems include chemical and physical changes in the plasma membrane, changes in the organization of cytoskeletal elements, susceptibility to retention of the second polar body, teratogenicity (yielding abnormal organisms), aneuploidy (yielding abnormal numbers of chromosomes), and chromosomal abnormalities due to spindle

disorganization or disruption. Fourth, information about the structure and ultrastructure of gametes and larvae that could help resolve the above problems is limited with respect to cryopreservation.

The goals of this work in eastern oysters were to: (1) evaluate the effect of extenders on the motility and fertilizing ability of sperm; (2) optimize cryopreservation techniques for gametes and larvae; (3) develop methods to evaluate the quality of gametes after cryopreservation, and (4) evaluate the performance of thawed sperm and larvae in the hatchery.

From the beginning, this project encountered a series of problems. The eastern oyster is a marine species and the Aquaculture Research Station, where the experiments were conducted, is ~250 km from the coast. Thus it was necessary to simulate natural conditions in the laboratory. Overall, holding systems were needed to maintain oysters, the use of artificial seawater (ASW) required evaluation and maintenance of large-scale algal cultures (~ 400 L) of Isochrysis galbana and Chaetoceros calcitrans as a food resource was required. Initially, oysters were transported in sacks from the coast. In the laboratory, the sacks were placed in commercial refrigerators. The oysters were used for experiments as soon as possible. This problem was addressed in Chapter 3 by performing experiments on the refrigerated storage of sperm and the use of extenders other than ASW. After recirculating holding systems were constructed, a constant supplement of ASW was available. Water from the oyster system was removed, placed in plastic trash cans (~100 L), sterilized with bleach and used to culture microalgae to feed larvae and adult oysters.

In Chapter 4, where the fertilizing ability of thawed sperm and survival of thawed larvae was investigated, it was necessary to perform fertilizations and to grow larvae.

The construction of systems to maintain ASW of good quality and the maintenance of algal cultures allowed to maintain larvae for a few weeks. However, there was no comparison between the results that could be obtained working in artificial conditions with those that could obtained in natural conditions. Therefore, in Chapter 5 It was tested the performance of thawed sperm and thawed larvae in an oyster hatchery on the Louisiana coast. The first problem here was the requirement of several million thawed larvae and several billion thawed sperm for use in the hatchery, in contrast with the smaller numbers used in the laboratory. Larger straws (5 mL) were used to increase storage capacity of sperm and larvae for use in these studies. These straws were transported frozen in shipping dewars to the hatchery. The hatchery lacked basic equipment, so, for example, it was used the kitchen and a research thermometer to produce desired thawing temperatures instead of a waterbath. Another problem when working in the hatchery was that the experiments were controlled by natural conditions, which had advantages and disadvantages. In this case, the studies were performed during the hurricane season and the first year's experiment was terminated early due to an approaching hurricane, necessitating repetition of the experiment the next year.

Two of the chapters in this dissertation have been published. Chapter 3 was published in the Journal of Shellfish Research (Paniagua et al. 1998). Chapter 5 represent the combination of a paper published in Cryo-Letters (Paniagua et al. 1998) and a book chapter to be published by the World Aquaculture Society (Paniagua et al. 2000).

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CHAPTER 2
INTRODUCTION

The species, Crassostrea virginica, was designated in 1985 as the "eastern oyster" by the Committee on Scientific and Vernacular Names of Mollusks of the Council of Systematic Malacologists (Turgeon, et al. 1988). It was necessary to revise the classification of oyster species to more accurately represent the diversity and taxonomic relationships within the bivalves (Harry 1985). Also, it was concluded that synonymization of names of oysters in several geographic regions was justified given that they were simply different populations of one species. For example, the eastern oyster which is distributed in the western Atlantic from Brazil northward through the Caribbean and Gulf of Mexico, including the Antilles, to the St. Lawrence River estuary in eastern Canada (a range of some 8,000 km), was known along its range as C. brasiliana, C. floridensis, C. guyenensis, C. lacerata, and C. rhizophorae. Thus, it was concluded that the names of these species should be subsumed by C. virginica (Harry 1985). Other works have suggested that due to the ease of hybridization and the homogeneity of chromosomal patterns, the designations of C. virginica, C. columbiensis and C. rhizophorae should be considered as comprising the same species (Menzel 1987). It is worth noting that invertebrate taxonomy can be contentious. Even the spelling of the word "mollusk" continues to be debated (Brusca and Brusca 1990). This dissertation will adhere to the recommendations of the second edition of the Common and Scientific Names of Aquatic Invertebrates from the United States and Canada: Mollusks (Turgeon, et al. 1988) unless otherwise noted.

Another controversy involves the use of the words "oocyte" and "egg". Oocytes have been defined as a gonial cell undergoing meiotic divisions. After completion of

meiosis and just before ovulation the oocyte is called an egg. This is a condition in mammals. However ovulation and fertilization can occur at different stages of meiosis depending on the species. For example, in oysters gonial cells resume meiotic maturation after fertilization and are said to be fertilized at prophase or at the first metaphase of meiosis. Thus, it could be asserted that oocytes become eggs some time after fertilization in oysters. However, given the uncertainty surrounding these terms, in this dissertation the term oocyte will be used for gonial cells within the gonad, and once the cells are removed from the gonad (either by natural spawning or by stripping) they will be referred to as "eggs".

The eastern oyster is a lamellibranch with pronounced bilateral asymmetry (Seed 1983). The eastern oyster begins life as a male and can later change sex to female (a condition called protandry) and usually spawns as a male in the first year. The germinal epithelium of the developing gonad consists initially of undifferentiated cells that develop into eggs and spermatocytes by the end of the first year. Eggs and sperm from adult oysters are spawned into seawater. Fertilized eggs begin division and in 8 to 12 h (depending on temperature) develop into free-swimming larvae or "trochophores" (50 to 60 μ in width). After ~24 h, the trochophore larvae develop into "veliger" or "D-stage" larvae (70 to 125 μ). Veliger larvae possess a shell in the form of two valves with a straight hinge. At this time a ciliated tuft enlarges and becomes organized as a ring around a projecting circular lobe (velum), which serves as the means of swimming and feeding. As the veliger larvae grow, their structure becomes more complex. The new features of note are the "umbo" (larval beak), which overhangs the previous straight hinge line, and the foot, with its retractor muscle, that allows the larvae to crawl. At this

stage the larvae are referred to as "pediveligers" (2 or 3 weeks) and remain planktonic. At about 48 h prior to settlement, the larvae swim with the foot slightly projecting from between the shell valves and begin to crawl on suitable surfaces for settlement. Attachment is followed by rapid extension of the shell. There is a marked transition between the larval prodissochonch (first double-shell), which follows the initial straight hinge shell, and the post-larva or dissoconch shell. Sudden growth at the shell margin completely changes the outward form of the newly settled oyster. Metamorphic changes are even greater within the body. Only a few hours after settlement the velum and foot disappear, the gill increases in size, the mouth moves to the adult position and the internal organs are reorganized. The settled larvae are referred to as "spat" and develop into adults within 1 to 2 years. Adult oysters mature and spawn gametes in the summer months (April to September in Louisiana) for the next cycle (Galtsoff 1964) (Fig. 2.1). Refrigerated and cryopreserved storage of gametes and larvae has been studied in vertebrates such as humans (Schneider 1986), mice (Bouquet et al. 1995) and fishes (Stoss 1983; Tom and Mitra 1994). However, few studies have addressed these topics in aquatic invertebrates.

Much of the work in this dissertation depended on accurate measurement and control of salinity. Thus it might be useful to review some terms here. Osmolality is defined as the number of particles (molecules, ions or atoms) per kg of water and is expressed as milliosmol per kg (mOsmol/kg). Salinity is defined as the total concentration of dissolved salts per kg of seawater and is expressed as the grams of salt per kilogram of seawater (g/kg) or parts per thousand (ppt). Osmolality is directly related

to salinity. For example full-strength seawater is 35 ppt and 800 mOsmol/kg. Half-strength seawater (brackish water) is around 15 ppt and 400 mOsmol/kg.

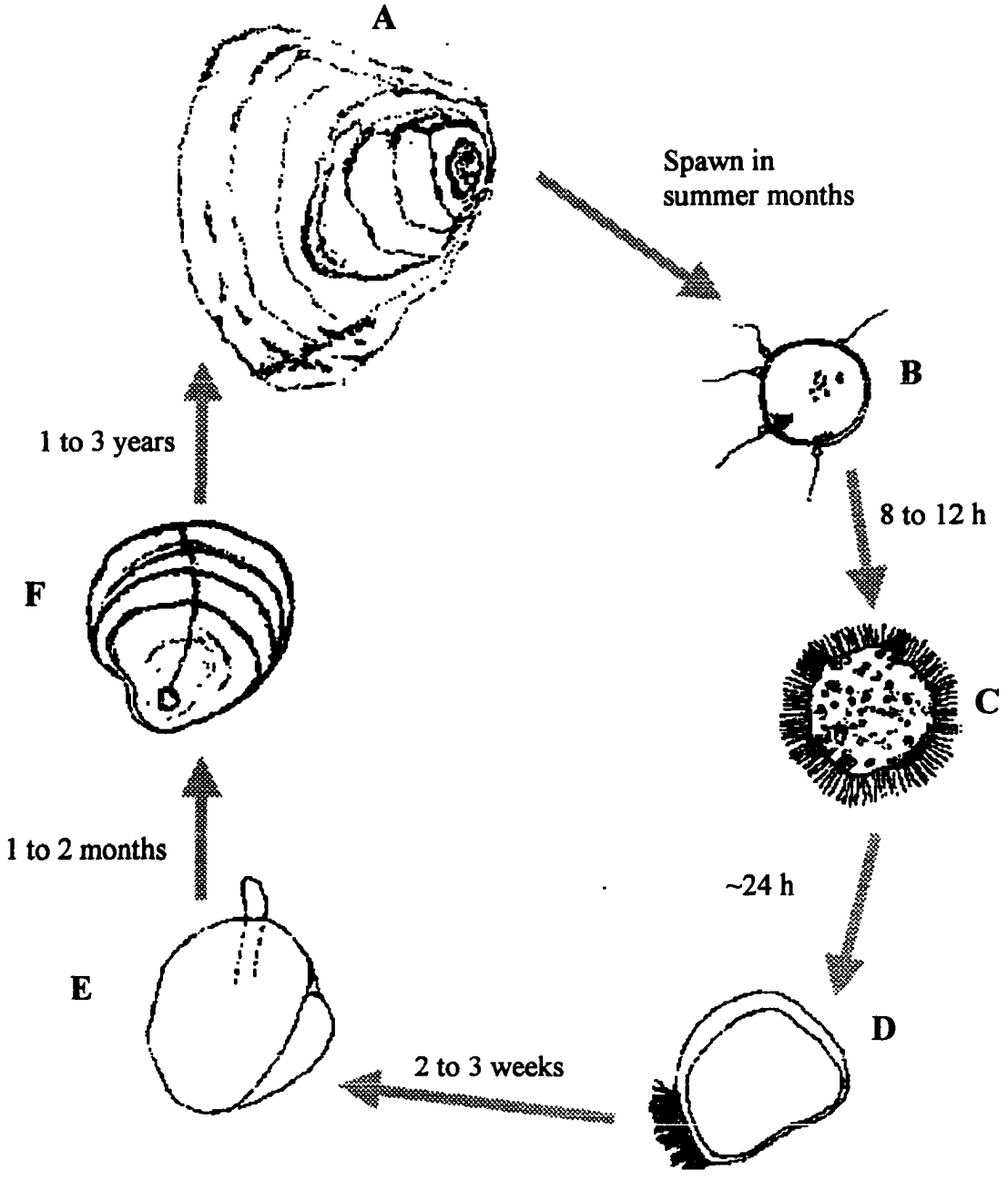

Figure 2.1 Life cycle of the eastern oyster Crassostrea virginica. A, adult oyster; B, fertilized egg surrounding by sperm; C, trochophore larvae; D, D-stage larvae; E, pediveliger larvae with "foot" extended; F, spat after settlement.

In the refrigerated storage of oyster sperm, factors such as osmotic pressure, sperm concentration, bacterial contamination, temperature, oxygen content, dissolved organic matter and pH are important. Control of these factors in other species has improved the success of refrigerated storage (Stoss et al. 1978). The factors influencing motility and fertilizing ability of oyster sperm during refrigerated storage had not been tested prior to this study. Hughes (1973) recognized the need to develop techniques for the frozen storage of oyster sperm and demonstrated morphological damage such as swollen heads, lost tails and low motility (between 1% and 5%) in response to different cryopreservation regimens. In these experiments, 70% of eggs exposed to fresh sperm were fertilized, while only 11% of eggs exposed to thawed sperm were fertilized. Problems such as unintentional elicitation of the acrosome reaction (deployment of the acrosome granule, a lysosome-like vesicle located at the tip of the sperm head) and sperm agglutination were observed. Zell et al. (1979) reported a technique for cryopreservation of sperm of C. virginica in the vapor of liquid nitrogen. Sperm were suspended in Hanks' salt solution containing 8% dimethyl sulfoxide (DMSO). After 68 d of storage, thawed sperm yielded 91% fertilization and after 11 d of incubation in fresh seawater, the larvae appeared normal.

Bougrier and Rabenomanana (1986) adapted methods of cryopreservation used for the marine fish, Dicentrarchus labrax, for use with the Pacific oyster, C. gigas. Seawater and DCSB4 buffer were used to suspend sperm, and 10% DMSO was used as the cryoprotectant. Sperm were frozen in a liquid nitrogen chamber and stored for 3 d. The qualitative scale (0 to 5) proposed by Legendre and Billard (1980) was used to estimate motility. Motility of thawed sperm varied from 1 to 3 (25% to 75%) and fertilization

rates with thawed sperm varied from 13% to 75%. Due to low fertilizing capacity of cryopreserved sperm, Kurokura et al. (1990) investigated the relation among survival, motility and morphology of thawed sperm to enhance cryopreservation. Decreased fertilizing capacity was due to sperm aggregation which decreased the availability of free sperm. Acrosome damage was the most frequent malformation in sperm and could be another mechanism that reduced fertilizing capacity. Yankson and Moyse (1991) recognized that cryobiological investigations should be performed in other species of bivalves. They reported the application of a simple cryopreservation recipe to four economically important species of oysters: Saccostrea cucullata, C. tulipa, C. Iredalei, and C. gigas. Different concentrations of DMSO (5%, 10%, 15% and 20%) in seawater containing 0.6% glycine were used and sperm were stored in liquid nitrogen for periods of from 12 to 217 days. The concentrations of DMSO yielding the highest fertility rates ranged from 10% to 20% for the four species.

Studies on the cryopreservation of oyster larvae and larvae are scarce. Renard (1991) studied the effects of methanol and sucrose and determined the cooling and freezing tolerances for C. gigas larvae. It was shown that the cooling tolerance to near freezing depended on the temperature and time of exposure and varied according to embryo source. The addition of sucrose enhanced slightly the cooling tolerance, and survival decreased as the storage temperature was lowered to -196°C. Gwo (1995) examined variables such as developmental stage, type and concentration of cryoprotectant, time of exposure to different cryoprotectants and cooling rates. The best survival (>60%) was found when trochophore larvae were incubated in 10% propylene glycol and were cooled at -2.5°C/min to -10°C before plunged into liquid nitrogen.

Cryopreservation of oyster eggs was studied by Chen et al. (1989). It was suggested that mortality was caused when eggs were incubated with 10% DMSO for 10 min, frozen in a computer-controlled freezer (two step method: from 20°C to -14 °C at a rate of 5°C/min, then to -30°C at a rate of 0.5°C/min), and thawed in a waterbath at 3°C. Information about the effects of cryoprotectants such as DMSO, ethylene glycol (EG), methanol, glycerol, sucrose and polyvinylpyrrolidone (PVP) on oyster eggs were reported by Liu and Robinson (1996). Sucrose and PVP at concentrations of less than 10% did not have toxic effects, while glycerol and methanol were highly toxic. The time of exposure to cryoprotectants was a critical factor in this experiment. The combination of DMSO and sucrose minimized egg mortality. The authors concluded that exposure to cryoprotectants before freezing can cause major biochemical and osmotic injuries to oyster eggs.

Assessment of the quality of gametes before and after cryopreservation is of great importance. Motility has been the method most used to assess viability of thawed sperm (Stoss and Holtz 1983). Freezing and thawing has been shown to produce severe impairment of cellular function in sperm of many species resulting in reduced fertility (Hammerstedt et al. 1990). In oysters, only one study has reported observations of survival, motility and morphology of thawed sperm (Kurokura et al. 1990). Recently, the use of flow cytometry has been used to evaluate quality of sperm in animals such as birds (Donoghue et al. 1995), mammals (Evenson et al. 1993) and fishes (McNiven et al. 1992). However, studies addressing the evaluation of oyster sperm have not been reported. Sperm is the most suitable type of gamete for analysis by flow cytometry. Eggs are often too large to move through a flow cytometer and can disrupt sample flow

by depositing lipids on the inside of the instrument. For the determination of egg quality before and after cryopreservation, other techniques such fluorescent microscopy may be helpful.

Most of the prior research in oysters has focussed on the Pacific oyster. The studies in this dissertation focussed on the eastern oyster due to the lack of information and the commercial importance of this species in Louisiana and elsewhere. Experiments were designed to optimize refrigerated storage and cryopreservation of sperm. The effects of extender solutions on sperm motility were examined and the best conditions for cryopreservation of larvae and eggs were determined. Also, male-to-male variability of fresh and thawed sperm was investigated and techniques were developed for evaluation of gamete quality. Finally, simplified methods for refrigerated storage and cryopreservation of sperm were described to assist application of cryopreservation in developing countries. It is hoped that the results found in this work will be of benefit to the oyster industry not only in the United States, but also in other countries where oysters are cultured.

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CHAPTER 3
EFFECT OF EXTENDER SOLUTIONS AND DILUTION ON MOTILITY AND FERTILIZING ABILITY OF EASTERN OYSTER SPERM

The eastern oyster, Crassostrea virginica, is an important resource of the Atlantic and Gulf coasts, and many studies have addressed culture of this species (e.g., Loosanoff and Davis 1963; Galstoff 1964; Dupuy et al. 1977). Artificial spawning techniques for various oyster species have been developed (Stephano and Gould 1988; Rampersad et al. 1994) and cryopreservation of sperm has been studied (Zell et al. 1979; Yankson and Moyse 1991). Although refrigerated storage of fish sperm is well studied (Stoss et al. 1978; Hara et al. 1982), studies of refrigerated storage of oyster sperm are lacking. Short-term storage of oyster sperm (for a few hours or days) can be useful for aquaculture. Stored samples can be transported from the spawning site to distant facilities for use in genetic studies and artificial breeding programs. Short-term storage offers the possibility of controlling factors that can affect sperm quality such as osmotic pressure and bacterial contamination. Another benefit of short-term storage and dilution of oyster sperm is the possibility of using this information for refinement of cryopreservation techniques.

Sperm quality is ultimately defined by the ability to fertilize eggs (Aas et al. 1991). Some factors that can influence the natural quality of oyster sperm are disease, environmental conditions, and genetic variability among males. In the short-term storage of oyster sperm, factors such as osmotic pressure, sperm concentration, bacterial contamination, temperature, oxygen content, dissolved organic matter, and pH are important. Control of these factors in other aquatic species has improved the success of refrigerated storage (Stoss et al. 1978). Sperm motility is a commonly used indicator of

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sperm quality and is usually expressed as the percentage of motile sperm after activation or on an arbitrary scale of from 0 to 5 (Redondo-Muller et al. 1991).

Among invertebrate species, the sea urchin has been used as a model for sperm study demonstrating that factors such as dilution and CO_2 concentration play an important role in motility intensity and duration (Gray 1928a; Gray 1928b). Few studies have examined the factors influencing motility of oyster sperm. Humphrey (1950) reported that oyster sperm suspended at less than 6×10^8 cells per mL rapidly lost motility. The effect of amino acids and other nutrients in prolonging the functional life of suspended spermatozoa has been examined. Glycine is utilized for energy production when the normal efficiency of the oxidative process is decreased (Jeffrey 1954a), and copper, zinc, and cadmium at low concentrations can be beneficial to oyster sperm motility (Jeffrey 1954b). Sea water (Yankson and Moyse 1991), Hanks' phosphate-buffered salt solution (Zell et al. 1979), and other buffers such as DCSB4 (Bougrier and Rabenomanana 1986) have been used successfully as extenders for cryopreservation studies for oyster sperm (with storage in buffers for 30 min or less).

The goal of this work was to evaluate extenders and dilutions used for refrigerated storage of oyster sperm. Our objectives were to: (1) compare motility of sperm stored undiluted or suspended in artificial seawater (ASW) of 5 osmotic pressures (22, 203, 403, 601, and 833 mOsmol/kg) during 24 h; (2) compare motility of sperm suspended in 833 mOsmol/kg solutions of ASW, Hanks' balanced salt solution (HBSS), and DCSB4 during 4 d of refrigerated storage; (3) compare motility of sperm stored for 4 d in ASW or HBSS (833 mOsmol/kg) at 6 ratios of sperm to extender (1:0, 1:1, 1:3, 1:8, 1:17 and 1:31), and (4) compare motility and fertilizing capacity of sperm stored in ASW at 200

or 833 mOsmol/kg, ASW with 6% glycine (ASW plus G) at 833 mOsmol/kg, and HBSS and calcium-free HBSS (C-F HBSS) at 830 mOsmol/kg (Paniagua et al. 1998).

MATERIALS AND METHODS

In this chapter, solution abbreviations will be followed by the osmolality of the solution. For example, an artificial seawater solution of 830 mOsmol/kg would be abbreviated as ASW 830.

Oyster collection

Oysters were obtained from a hatchery on Grand Isle, Louisiana maintained by the Louisiana Sea Grant College Program, and were transported to the Louisiana State University Agricultural Center, Aquaculture Research Station, Baton Rouge. Oysters were opened and inspected visually for the presence of gonad development and prominent genital canals (Supan 1996). A gonad sample was collected with a capillary tube and smeared on a glass microscope slide for examination at 20 X objective. Sex was identified based on the presence of eggs or sperm.

Gamete preparation

Gamete samples were removed from each oyster by the dry stripping method (Allen and Bushek 1992). The gonad was gently disrupted and gonadal material collected with a Pasteur pipette. A 10-μL sample was removed from the gonad to measure osmolality with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah). Egg or sperm samples were placed in 50-mL beakers until suspension in an extender. After suspension, eggs were washed through a 70-μ nitex screen (Aquacenter, Leland, Mississippi), collected on a 15-μ screen, and suspended in ASW 601. For sperm, samples were washed through 70-μ and 15-μ screens and motility was estimated as described below.

Motility estimation

A 10-μL sample was removed from sperm suspensions to estimate motility. The sample was mixed with 20 μL of ASW 601 on a glass microscope slide. The percentage of sperm exhibiting vigorous forward movement was estimated at 20X objective using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, New York). Sperm vibrating in place were not considered to be motile. Only males with actively swimming sperm (> 90%) were selected for experimentation.

Throughout the experiments, five extender solutions were used: ASW (Fritz Super Salt, Fritz Industries, Inc. Dallas, Texas), ASW with 6% glycine, HBSS (Tiersch et al. 1994), C-F HBSS, or DCSB4 (Bougrier and Rabenomanana 1986). All chemicals (except ASW) were of reagent grade (Sigma Chemical Corp., St. Louis, Missouri).

Experiment 1: Sperm motility in artificial seawater of different osmotic pressures

Sperm samples were collected from three males (initial motility was 99% for all samples). Aliquots of 250 μL from each sample were suspended in ASW of 22, 203, 403, 601, or 833 mOsmol/kg. Osmolality of suspensions was measured with a vapor pressure osmometer. The final volume of each sperm suspension was adjusted to 3.0 mL, and the sperm concentration was estimated with a Makler sperm counting chamber (Sefimedical Instruments, Haifa, Israel). Samples (10-μL) were examined for sperm motility every 4 min for 1 h and once every h for the following 2 h. To determine if sperm suspended in ASW 22 and 203 could regain motility, 10-μL samples were diluted with 20 μL of fresh ASW 833 and incubated for 5 min. After these observations, sperm were refrigerated at 4^0C. Sperm motility was estimated again at 24 h after warming to room temperature (21^0C).

Experiment 2: Sperm motility in artificial seawater, Hanks' balanced salt solution, and DCSB4 solution

Based on Experiment 1, osmolality was set at 833 mOsmol/kg for subsequent experiments. Sperm samples from three males were suspended in ASW, HBSS or DCSB4 using the procedures described above. Concentrations were adjusted to 1 x 10^8 sperm per mL and the suspensions were refrigerated at 4^0C. Motility was monitored daily for 4 d. All suspensions were allowed to warm to room temperature before motility assessment.

Experiment 3: Sperm motility in artificial seawater and Hanks' balanced salt solution in five dilutions

Sperm samples from three males were stored undiluted, or suspended in ASW 833 or HBSS 833 at six ratios of sperm to extender (1:0, 1:1, 1:3, 1:7, 1:17 and 1:31). As in Experiment 2, samples were refrigerated and motility was monitored daily for 4 d.

Experiment 4: Sperm motility and fertilizing ability in five extenders

Sperm samples from three males were stored undiluted, or suspended in the following extenders: ASW 200, ASW 833, ASW plus G 833, HBSS 830, and C-F HBSS 830. Sperm motility was estimated after suspension for 5 min. Eggs of three ripe females were used to determine the fertilizing ability of sperm suspended in the different extenders. Approximately seven thousand eggs (35 eggs per mL) were suspended and incubated for 1 h in 200 mL of ASW 830 in 400-mL plastic beakers (VWR Scientific Inc. St. Louis, Missouri). Sperm concentrations were estimated with a Makler counting chamber and the final concentration was set at 5 x10^4 sperm per mL. Concentration of eggs was determined by counting of 1-mL aliquots in a Sedgewick-Rafter chamber (Hausser Scientific Parthership, Horsham, Pennsylvania). Twelve h after fertilization, the

number of trochophore larvae per mL was estimated with a Sedgewick-rafter chamber. Gamete and larvae counts were performed in duplicate.

Data analysis

Statistical analysis was performed using SAS software for Windows ® (SAS Institute, Cary, North Carolina). Data from Experiments 1, 2, and 3 were not distributed normally. For these experiments, a non-parametric Friedman's test was used to test the association among extenders, percent motility, and duration of motility, and to test for significant differences. Data from Experiment 4 were distributed normally and treatment groups were compared using a one-way factorial analysis (ANOVA). Specific differences among treatment groups were identified by the least squares differences test. A value of $P < 0.05$ was chosen as the level for significance.

RESULTS

Experiment 1: Sperm motility in artificial seawater of different osmotic pressures

Sperm motility in ASW of different osmotic pressures was significantly different ($P = 0.001$) (Table 3.1). The osmolality of the gonad was 573 $\pm$ 45 mOsmol/ kg (mean $\pm$ SD). Sperm suspended in ASW 833 retained 99 $\pm$ 0% motility for 2 h (Fig. 3.1). Sperm suspended in ASW 403 and ASW 601 had an initial motility of less than 30 $\pm$ 3% followed by an increase to 90 $\pm$ 20% or higher after 8 min. This motility was retained for 2 h. Sperm suspended in ASW 203 showed low motility (~1 %) in the first 4 min; the maximum motility after 16 min of incubation was 47 $\pm$ 5 % and was retained for 1 h. Subsequent dilution of these samples in ASW 833, did not increase motility. No motility was observed in sperm suspended in ASW 22 initially or during the 3-h experiment. After cold storage for 24 h, the highest motilities were 75 $\pm$ 7% for sperm suspended in ASW 833 and 80 $\pm$ 10% for sperm suspended in ASW 601. Sperm in ASW 403 showed

a decreased motility (to 40 ± 15%), and sperm in ASW 203 had the greatest reduction in motility (to <10%).

Table 3.1 Eastern oyster sperm were suspended in artificial seawater at osmolalities of 22, 203, 403, 601 or 833 mOsmol/kg and motility was analyzed using a non-parametric Friedman's test for the effect of osmolality.

Statistic	Alternative hypothesis	DF	Value	Probability
1	Non-zero correlation	1	9.503	0.002
2	Raw mean scores differences	17	70.332	0.001
3	General association	255	340.968	0.001

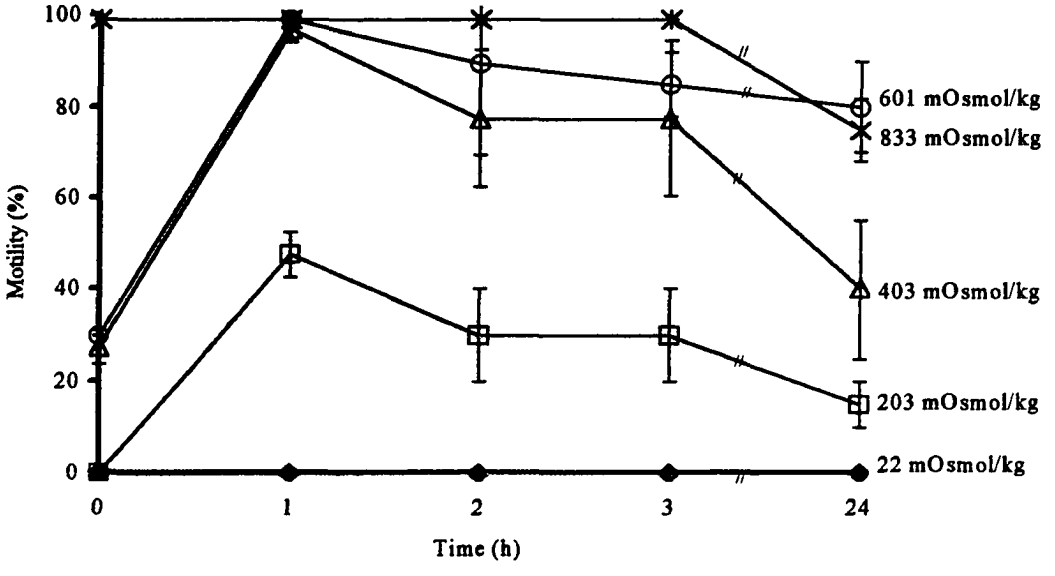

Figure 3.1 Percent motility (mean ± SD) of eastern oyster sperm suspended in artificial seawater at osmolalities of 22, 203, 403, 601, or 833 mOsmol/kg (n = 3 for each treatment).

Experiment 2: Sperm motility in artificial seawater, Hanks' balanced salt solution, and DCSB4 solution

Cold storage of oyster sperm in different extenders (dilution 1:3) revealed no significant differences ($P = 0.267$) (Table 3.2). The highest initial motility was 85 ± 4% for sperm suspended in ASW 833, and the lowest was 73 ± 3% for sperm suspended in

DCSB4 833. By day 3, sperm suspensions in each of the three extenders had motility values of <10% (Fig 3.2). Bacteria were observed during motility estimates after 1 d of storage. Bacterial concentrations increased with time and were associated with decreased sperm motility. Preliminary identification of these bacteria identified the presence of Vibrio species. (A. Camus and J. Hawke, Louisiana State University Aquatic Animal Diagnostic Laboratory, personal communications).

Table 3.2 Eastern oyster sperm were stored at 4°C and motility was analyzed using a non-parametric Friedman's test for the effect of diluent.

Statistic	Alternative hypothesis	DF	Value	Probability
1	Non-zero correlation	1	1.714	0.191
2	Raw mean scores differences	2	5.083	0.079
3	General association	48	10.000	0.267

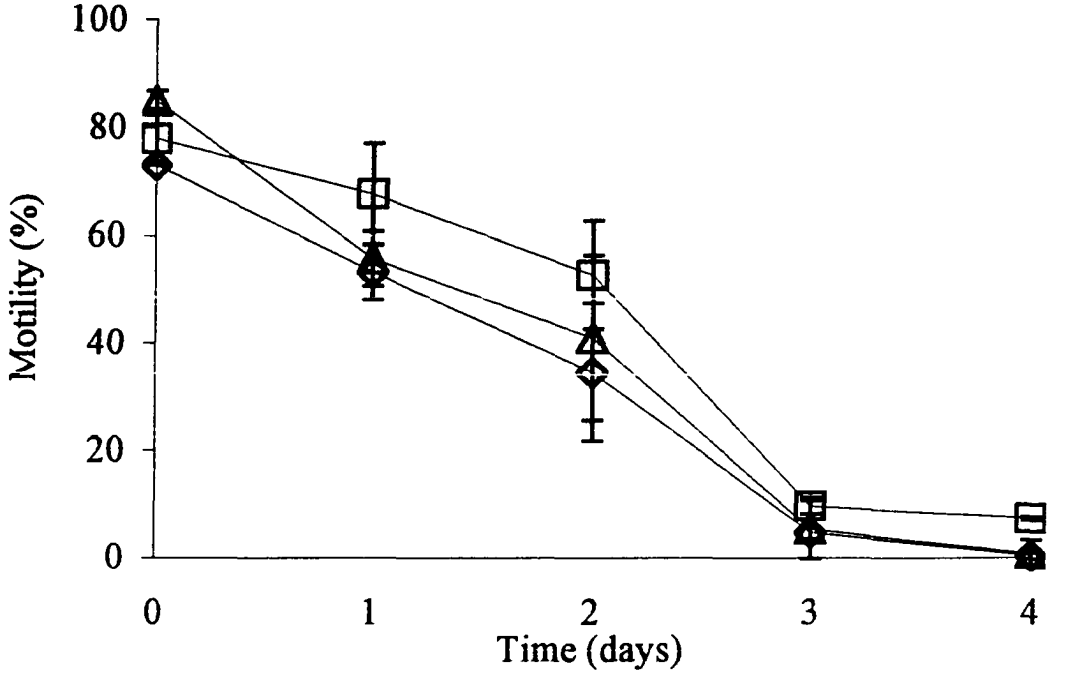

Figure 3.2 Percent motility (mean ± SD) of eastern oyster sperm stored at 4°C in DCSB4 solution (diamonds), Hanks' balanced salt solution (HBSS; squares), or artificial seawater (ASW; triangles) at 833 mOsmol/kg (n = 3 for each treatment).

Experiment 3: Sperm motility in artificial seawater and Hanks' balanced salt solution in five dilutions

Significant differences in sperm motility were found among different dilutions ($P = 0.001$) (Table 3.3). For ASW 833, undiluted sperm had the highest motility (96 ± 5%) followed by sperm diluted at 1:1 (86 ± 11%) (Fig 3.3). While sperm diluted at 1:31 had the lowest motility (47 ± 15%). After 3 days, sperm samples diluted at 1:1, 1:3, or at 1:7 retained <10% motility. After 4 days, all samples display a complete loss of motility. For HBSS 833, undiluted sperm had the highest initial motility (95 ± 6%) followed by sperm diluted at 1:7 (90%) (Fig. 3.4). Sperm diluted at 1:31 had the lowest motility (40 ± 14%). After 1 day of storage, sperm diluted at 1:31 had a complete loss of motility. Overall, motility was maintained best in less dilute samples. For undiluted samples, motility never declined below 50% (Figs. 3.3 and 3.4). As in Experiment 2, bacteria were observed in all samples and increased with time and were associated with decreased motility.

Table 3.3 Eastern oyster sperm were suspended in artificial seawater and Hanks' balanced salt solution (833 mOsmol/kg), stored at 4°C and analyzed using a non-parametric Friedman's test for the effect of dilution (sperm to extender: 1:0, 1:1, 1:3, 1:7, 1:15 or 1:31) on motility

Statistic	Alternative hypothesis	df	Value	Probability
1	Non-zero correlation	1	43.359	0.001
2	Raw mean scores differences	5	74.406	0.001
3	General association	0	155.590	0.001

Experiment 4: Sperm motility and fertilizing ability in five extenders

Sperm motility was affected by extender ($P = 0.0001$) (Table 3.4). The highest motility was observed for sperm suspended in C-F HBSS 830 (96 ± 5%) and the lowest motility was for sperm suspended in ASW 200 (12 ± 8%) (Fig. 3.5).

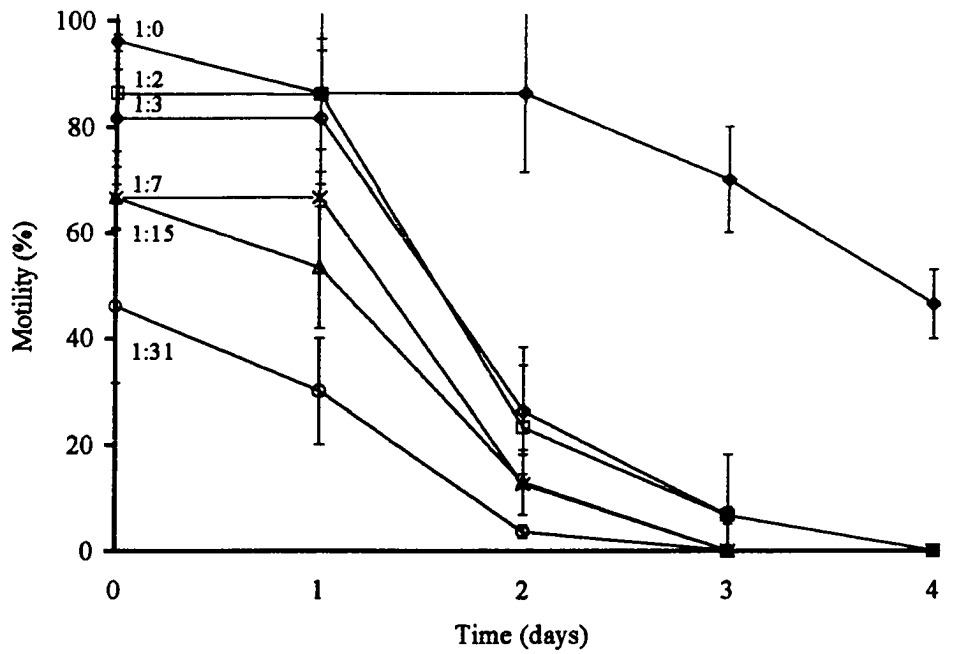

Figure 3.3 Percent motility (mean ± SD) of eastern oyster sperm suspended in artificial seawater (833 mOsmol/kg) at six ratios of sperm to extender (n = 3 for each treatment).

Table 3.4 Eastern oyster sperm were suspended in five diluents and analyzed using an one-way analysis of variance for the effect of diluents on motility.

Source of variation	df	SS	F	P
Diluents	4	2.460	42.18	0.001
Error	10	0.146	--	--
Corrected total	14	2.607	--	--

Agglutination of sperm, causing adherence and sperm immobilization, was evident in samples suspended in ASW 200 and ASW 830. The extender used to dilute sperm had

an effect on fertilization as evaluated by larval development at 12 h ($P = 0.0001$) (Table 3.5). Pair-wise comparisons showed significant differences except for ASW 833 and ASW plus G833. Examination of trochophore larvae at 12 h after fertilization indicated highest fertilization (48%) with sperm diluted in C-F HBSS, and the lowest fertilization (<1%) with ASW 200 (Fig. 3.5).

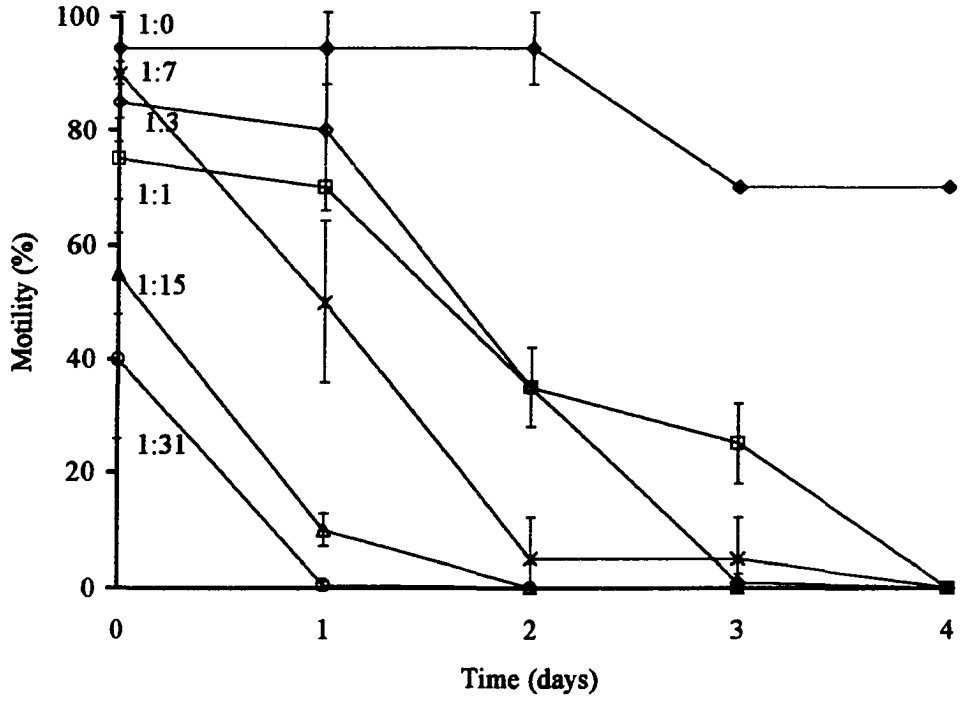

Figure 3.4 Percent motility (mean ± SD) of eastern oyster sperm suspended in HBSS (833 mOsmol/kg) at six ratios of sperm to extender (n = 3 for each treatment).

Table 3.5 Survival of trochophore larvae obtained from fertilization by sperm suspended in five diluents was analyzed using an one-way analysis of variance for the effect of diluents on fertilizing ability.

Source of variation	df	SS	F	P
Diluents	4	0.494	19.84	0.001
Error	10	0.062	--	--
Corrected total	14	0.556	--	--

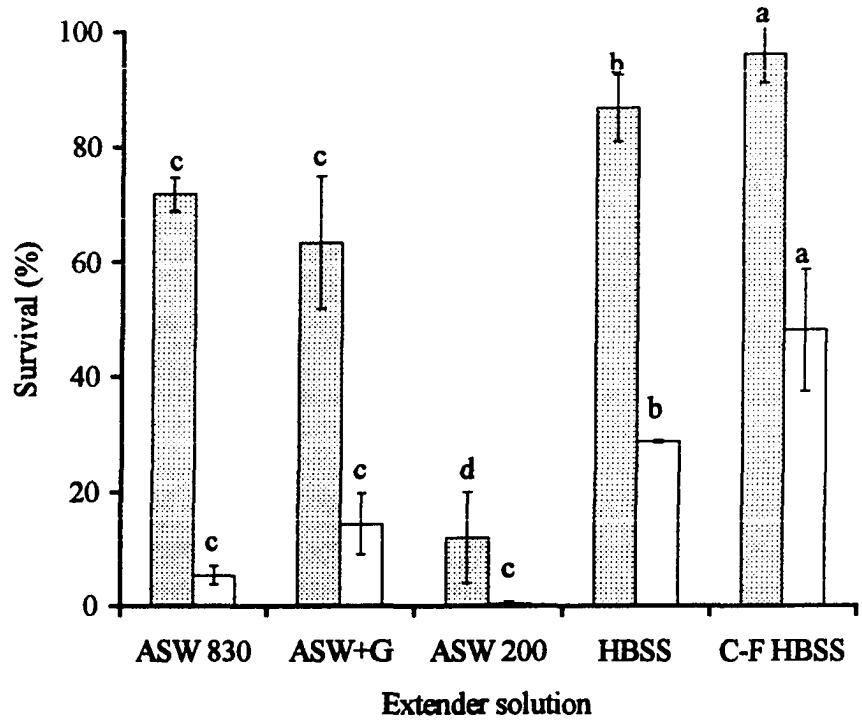

Figure 3.5 Percent survival (mean ± SD) of trochophore larvae (12 h after fertilization) obtained from fertilization by sperm suspended in artificial seawater at 830 mOsmol/kg (ASW 830), artificial seawater at 830 mOsmol/kg plus glycine (ASW plus G), artificial seawater at 200 mOsmol/kg (ASW 200), Hanks' balanced salt solution at 830 mOsmol/kg (HBSS), or calcium-free HBSS at 830 mOsmol/kg (C-F HBSS) (n = 3 for each treatment). Shaded bars represent sperm motility; open bars, larval survival.

DISCUSSION

Sperm can be active in the gonad of marine invertebrates such as oysters (Loosanoff and Davis 1963). When sperm are released in the water they may accelerate, decelerate or cease moving in response to various stimuli. Factors such as salinity, temperature, oxygen content, dissolved organic matter and pH can affect the fertilizing ability and motility of oyster sperm when released into natural seawater (Humphrey 1950). Another factor that can affect motility is osmotic pressure. In this study, sperm motility increased with osmolality and time of exposure. Sperm suspended in relatively high osmolalities (>400 mOsmol/kg) did not lose motility. After 24 h of storage, sperm retained high motility (~ 85%) suggesting that cold storage of sperm at high osmolalities is possible

without significant loss of motility. Factors that could affect sperm motility in dilute ASW are osmotic shock and imbalances of ions necessary to establish motility. It is possible that sperm suspended in diluted ASW (less than 400 mOsmol/kg) could require more than 8 min (our observation period) to adjust to the new osmotic pressure and acquire maximum motility. The addition of fresh ASW 833 did not reactivate sperm initially suspended in ASW 22 suggesting that sperm undergoing strong changes in osmotic pressure could be irreversibly damaged. The effect of ions such as potassium sodium, calcium or magnesium on motility remains to be examined.

Sperm motility declined with time when dilutions were made with DCSB4, HBSS and ASW. This loss of motility could be due to exhaustion of metabolic energy reserves with continuous activity after suspension, or to detrimental effects caused by bacteria. In refrigerated storage of fish sperm, bacterial growth can be a major problem (Jenkins and Tiersch 1997). In this study, declining motility was associated with increased bacterial numbers. According with the clinical report, mixed vibrios and other unidentified species were found in the samples. Addition of antibiotics could remedy this problem (Scott and Baynes 1980). The potential treatment recommended by the LSU Aquatic Animal Diagnostic Laboratory was neomycin and polymyxin at 10 μg per mL.

Dilution is another possible cause for reduced sperm motility. In the oyster it has been noted that the greater the dilution, the greater the sperm activity in the first minutes (Gray 1928a). The loss of motility during storage due to dilution could be because sperm are less crowded in the suspension and have more free space in which to move, causing a depletion of energy, or because essential factors in the gonad are lost when sperm is diluted.

Studies have shown that when sea urchin sperm were suspended in seawater or ASW, they underwent a spontaneous acrosome reaction consisting of exocytocis of the acrosome granule, which is a lysosome-like vesicle located at the tip of the sperm head (Gonzalez-Martinez et al. 1992). Spontaneous acrosome reaction could in part be responsible for loss of fertilizing ability. In calcium-free artificial seawater, sea urchin sperm showed no morphological changes and swam vigorously (Dan 1955). In the presence of calcium, the sperm underwent the acrosome reaction and became agglutinated.

Oyster sperm, like those of the sea urchin, possess an acrosome (Galtsoff and Philpott 1960; Bozzo et al. 1993) which plays an important role in fertilizing capacity. Cryopreserved oyster sperm may have lost fertilizing capacity due to damage to the acrosome, a lack of capacity to trigger cleavage, preventing entry of sperm into eggs (Kurokura et al. 1990). In the present study, sperm diluted in C-F HBSS reached a high motility (99%) and did not agglutinate, suggesting that the acrosome reaction was minimized. Also, trochophore numbers were highest with sperm suspended in C-F HBSS. Glycine has been shown to enhance motility of oyster sperm under certain conditions (Jeffrey 1954a); however, no benefit of glycine was found for sperm motility or larval survival in this study. Sperm had diminished motility when placed in ASW 200, and there was no fertilization, suggesting that the sperm lost fertilizing ability due to damage caused by osmotic shock or a change in ionic composition.

Several conditions were found to influence the motility and fertilizing ability of eastern oyster sperm in this study and these results can be of practical use for refrigerated storage and cryopreservation. Osmotic pressure should be maintained at a high level

(> 400 mOsmol/kg) to ensure good motility, and the use of antibiotics should be investigated. When sperm is to be used within 24 h, a low dilution (1:1 or 1:3) is recommended; however, for more than 1 d of refrigerated storage, samples should be maintained undiluted. In addition, suspension of sperm in C-F HBSS appears to have a positive effect on sperm motility and fertilizing ability, and could enhance larval production.

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CHAPTER 4
LABORATORY STUDIES OF CRYOPRESERVATION OF OYSTER SPERM AND LARVAE

Freezing of sperm is not a new technology. It dates back over 50 years when Polge (1949) discovered that the addition of glycerol allowed survival of bovine sperm after thawing. Today, cryopreservation techniques in domestic animals are well known and applied in commercial fields such as the dairy industry (Fahning and Garcia 1992). In aquatic animals, the most successful use of cryopreservation has been for fish sperm. To date, spermatozoa of over 200 species of freshwater and marine fishes have been cryopreserved (Rana 1995). The majority of publications and protocols relate to three groups of fish of aquacultural importance: salmonids, tilapia and carps (Rana 1995). In contrast to the extensive research and advances made in cryopreservation of sperm of domestic animals, few methodologies are available for the application of cryopreservation to aquatic organisms other than fish. Research in aquatic invertebrates has concentrated on spermatozoa of the Pacific oyster, Crassostrea gigas (McFadzen 1995).

Twenty five years after the report by Polge, successful cryopreservation of bovine embryos was published (Fahning and Garcia 1992). After this achievement, successful cryopreservation of embryos from other domestic animals such as cow, sheep, goat, pig and horse were documented (Table 4.1). In aquatic organisms, little research has been reported on the cryopreservation of embryos and larvae.

In the United States, the most important oyster species is the eastern oyster, Crassostrea virginica. In Louisiana, production of the eastern oyster has an approximate annual farm value of $50 million (Avery 1998). Despite the benefits of the techniques of cryopreservation, little research has been done with the eastern oyster. There are two reports on the cryopreservation of eastern oyster sperm performed more than 20 years ago (Hughes 1973; Zell et al. 1979). There are no reports on cryopreservation of embryos or larvae of the eastern oyster.

The goal of this work in the eastern oyster was to develop cryopreservation techniques for sperm and larvae in the laboratory for subsequent use in the hatchery. The objectives were to: (1) optimize techniques for cryopreservation of sperm; (2) evaluate the

effect of variability of thawed sperm from individual males on fertilizing ability, and (3) develop techniques for cryopreservation of trochophore larvae.

Table 4.1 Cryopreservation of embryos of some domestic animals

Organism	Pregnancy rate	Year	Reference
Cow	44	1978	Lehn-Jensen and Greve
Cow	74	1986	Christie
Cow	67	1995	Hasler et al.
Sheep	57	1984	Tervit and Goold
Sheep	54	1986	Heyman
Sheep	73	1993	McGinnis et al.
Goat	68	1986	Chemineau et al.
Goat	50	1988	Wang et al.
Pig	0	1985	Nieman
Pig	45	1989	Hayashi et al.
Pig	12	1991	Kashiwazaki et al.
Horse	50	1984	Takeda et al.
Horse	53	1985	Slade et al.

MATERIALS AND METHODS

Oyster collection, gamete preparation and motility estimation

Oyster collection, gamete preparation, and motility estimation were performed as described in standard operational procedures (SOP) (Appendix A).

Pre-freezing treatment. Sperm from three males (96 ± 5% motility) was used for the experiment. Sperm samples were diluted in calcium-free Hanks' balanced salt solution (C-F HBSS) (676 mOsmol/kg, pH 7.5) according to SOP (Appendix A). Osmotic pressure was measured with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah). Cryoprotectant solutions (CPS) were prepared with fresh-made filtered C-F HBSS containing 0%, 5%, 10%, 15%, 20%, or 25% (v/v) propylene glycol (PG) for a

final volume of 30 mL (Table 4.2). Another series of CPS was prepared by adding 0.25M sucrose to the solutions. Sperm suspensions (15 mL) were placed in plastic weighing boats (VWR Scientific Inc., St. Louis Missouri) and CPS was added to a final volume of 30 mL. Sperm aliquots (5 mL) were placed in 5-mL macrotubes (Minitube of America, Inc., Madison, Wisconsin) and equilibrated for 20 min at 21°C. Osmotic pressure and motility was measured after the addition of CPS.

Freezing. After equilibration, sperm were frozen in a controlled-rate freezer (Kryo 10 series II, Planer products, Sunbury-on-Thames, United Kingdom). The initial temperature was 15°C, the samples were cooled at a rate of –2.5°C per min until reaching a final temperature of -30°C which was held for 5 min (Fig. A.1). Tubes were plunged into liquid nitrogen and held for 2 weeks.

Table 4.2 Preparation of cryoprotectant solutions (CPS) for eastern oyster sperm.

CPS concentration (%)	C-F HBSS (mL)	Propylene glycol (mL)	Sperm volume (mL)	Final volume (mL)
0	15.0	0.0	15.0	30.0
5	13.5	1.5	15.0	30.0
10	12.0	3.0	15.0	30.0
15	10.5	4.5	15.0	30.0
20	9.0	6.0	15.0	30.0
25	7.5	7.5	15.0	30.0

Thawing. Tubes were thawed in a waterbath at 25°C for 30 sec or at 70°C for 15 sec. After thawing, sperm samples (5 mL) were placed in 50-mL beakers (VWR Scientific Inc.) and an equal volume of C-F HBSS was added. Motility and osmotic pressure were estimated after dilution in fresh C-F HBSS.

Fertilization. Eggs of three ripe females were used to determine the fertilizing ability of thawed sperm. Egg concentration was determined as described in Chapter 3. A suspension containing approximately 304,000 eggs per mL was incubated in artificial seawater (ASW) for 1 h in a 500-mL plastic beaker. The osmotic pressure of the egg suspension was 512 mOsmol/kg. Approximately 14,000 eggs (35 eggs per mL) were placed in a 500-mL plastic beaker (VWR Scientific Inc.) and fertilized with 500 μL of thawed sperm (~ 5 x 10^8 sperm per mL). A control replicate was used to test the fertilizing ability of fresh gametes. Eggs from the same stock solution were fertilized with fresh sperm (> 90% motility) suspended in C-F HBSS (679 mOsmol/kg). After fertilization, sterilized ASW (699 mOsmol/kg, pH 7.5) was added to yield a final volume of 400 mL. The samples and control were incubated at room temperature (25°C) for 12 h. After incubation, the number of larvae per mL was counted in a Sedgewick-Rafter chamber. All gamete and larvae counts were performed in duplicate.

Thawed sperm variability from individual males

Fertilizing ability of sperm before cryopreservation. Gametes from five females and five males (> 90% motility) were placed in individual plastic beakers. Sperm samples were suspended in C-F HBSS (641 mOsmol/kg). The total volume per male was 6 mL and mean sperm concentration was 4.4 $\pm$ 1.4 x 10^8 per mL. Eggs from individual females were placed in separate 500 mL plastic beakers in concentrations of 35 eggs per mL. Eggs from each female were fertilized with sperm of each of the five males in a 5 x 5 fertilization matrix. To compare the fertilizing ability of individual males from pooled males, eggs from the five females were mixed in the same proportions yielding a total concentration of 35 egg per mL. Fertilization, incubation and larvae counts were made as described above.

Freezing and thawing. Sperm suspensions from the individual males were placed in plastic boats, and 15% propylene glycol was added to yield a final volume of 5 mL. Sperm aliquots were placed in 5-mL macrotubes and equilibrated for 20 min at 21°C. After equilibration, the macrotubes were frozen in a controlled-rate freezer in the same conditions as described above. Tubes were plunged in liquid nitrogen and after a month of storage the straws were thawed at 70°C and sperm were diluted (1:1) in fresh-made C-F HBSS.

Fertilizing ability of thawed sperm. Eggs from five ripe females (different than the females used in the fertilization experiment described above) were used to assess the fertilizing ability of thawed sperm. Eggs from each female were fertilized with thawed sperm of each of the five males yielding a 5 x 5 fertilization matrix. To test the quality of eggs, fresh sperm samples of five males were pooled in the same proportions as those used for individual males and were used to fertilize eggs from the five females. Fertilized eggs were incubated for 12 h at room temperature (25°C). After incubation, the number of larvae per mL was counted in a Sedgewick-Rafter chamber. All gamete and larvae counts were performed in duplicate.

Cryopreservation of trochophore larvae

Fertilization. Approximately six million eggs (35 eggs per mL) were suspended in 2 L of ASW and fertilized with sperm in a ratio of 1 egg to 5 or 10 sperm. Concentrations of eggs were determined by counting of 1-mL aliquots in a Sedgewick-Rafter chamber. Fertilized eggs were incubated until ~50% were in first cell division at 25°C (~1 h). After incubation, fertilized eggs were placed in 15-L plastic buckets at 21°C. Twelve h after fertilization, the number of trochophore larvae per mL was counted in a Sedgewick-Rafter chamber. Gamete and larvae counts were performed in duplicate. Temperatures varied

during the experiment based on the location of specific activities. Fertilizations were performed in the wet laboratory at 25°C and larvae were incubated on the laboratory bench (21°C) until cryopreservation at 12 h after fertilization (trochophore stage).

Pre-freezing and freezing. The cryoprotectant solutions (CPS) were prepared based on the procedures of Gwo (1995). Two CPS were prepared with filtered ASW containing 10% or 15% propylene glycol (PG). Trochophore larvae were concentrated on a 30-μ nitex screen yielding a total volume of 24 mL (3 million larvae). After concentration, the larvae were mixed with CPS. Five different concentrations (10, 100, 1000, 10,000, and 100,000 larvae per mL) were tested for each CPS (Table 4.3). Osmotic pressure of the suspensions was measured before (642 mOsmol/kg) and after addition of CPS (1,461 mOsmol/kg for larvae suspended in 10% PG and 1,627 mOsmol/kg for larvae suspended in 15% PG). Five-mL aliquots were placed in 5-mL macrotubes and equilibrated for 20 min at 21°C. After equilibration, the macrotubes were frozen in a controlled-rate freezer using the conditions described in Appendix A.

Table 4.3 Preparation of cryoprotectant solutions for eastern oyster larvae. CPS: cryoprotectant solution; ASW: artificial seawater, and PG: propylene glycol.

		CPS (10%)		CPS (15%)		
Larvae concentration per mL	Larval suspension added (μL)	ASW (mL)	PG (mL)	ASW (mL)	PG (mL)	Total volume (mL)
10	0.8	8.999	1.0	8.499	1.5	10
100	8	8.992	1.0	8.492	1.5	10
1000	80	8.920	1.0	8.420	1.5	10
10,000	800	8.200	1.0	7.700	1.5	10
100,000	8000	1.000	1.0	0.500	1.5	10

Thawing. Tubes were thawed in a waterbath at 70°C for 15 sec. After thawing, the larvae were placed in 50-mL plastic beakers containing 5 mL of filtered ASW. The number of motile trochophore larvae per mL was estimated by counting in a Sedgewick-Rafter chamber.

Data analysis

Statistical analysis was performed using SAS software for Windows® (SAS Institute, Cary, North Carolina). Motility of sperm was analyzed using a three-way analysis of variance for the effect of cryoprotectant concentration, presence or absence of sucrose, and thawing temperature. Fertilizing ability of sperm was analyzed using a three-way analysis of variance for the effect of cryoprotectant presence or absence of sucrose, and thawing temperature. One-way factorial analysis of variance was used to test male-to-male variability. For frozen larvae, two-way analysis of variance was used to test the effect of concentration of larvae per straw and cryoprotectant concentration. Data were arcsine square-root transformed before analysis. Specific differences among treatment groups were identified by the least square difference test. A value of $\underline{P} < 0.05$ was chosen as the level for significance.

RESULTS

Osmotic pressure and sperm motility

Sperm suspended in CPS with sucrose lost motility faster than did sperm suspended in CPS without the addition of sucrose (Figure 4.1). Sperm suspended in 0, 5%, 10% and 15% propylene glycol without addition of sucrose maintained a high motility (> 90%)

Motility and fertilizing ability of thawed sperm

For sperm thawed at 25°C, the highest motility (13%) was found for sperm suspended in 15% propylene glycol with the addition of sucrose. The highest fertilization (29%) was

found for sperm suspended in 10% propylene glycol (Figure 4.2). For sperm thawed at 70°C, the highest motility (22%) was found for sperm suspended in 15% propylene glycol with the addition of sucrose, and the highest fertilization (60%) was found for sperm suspended in 10% propylene glycol (Figure 4.3).

Sperm motility and fertilizing ability was affected by the concentration of cryoprotectant ($\underline{P} = 0.0001$) (Tables 4.4 and 4.5). Addition of sucrose did not enhance sperm motility ($\underline{P} = 0.9020$) (Table 4.4) or fertilizing ability ($\underline{P} = 0.4314$) (Table 4.5). Thawing rate had an effect on sperm motility ($\underline{P} = 0.0163$) (Table 4.4) and fertilizing ability ($\underline{P} = 0.0145$) (Table 4.5).

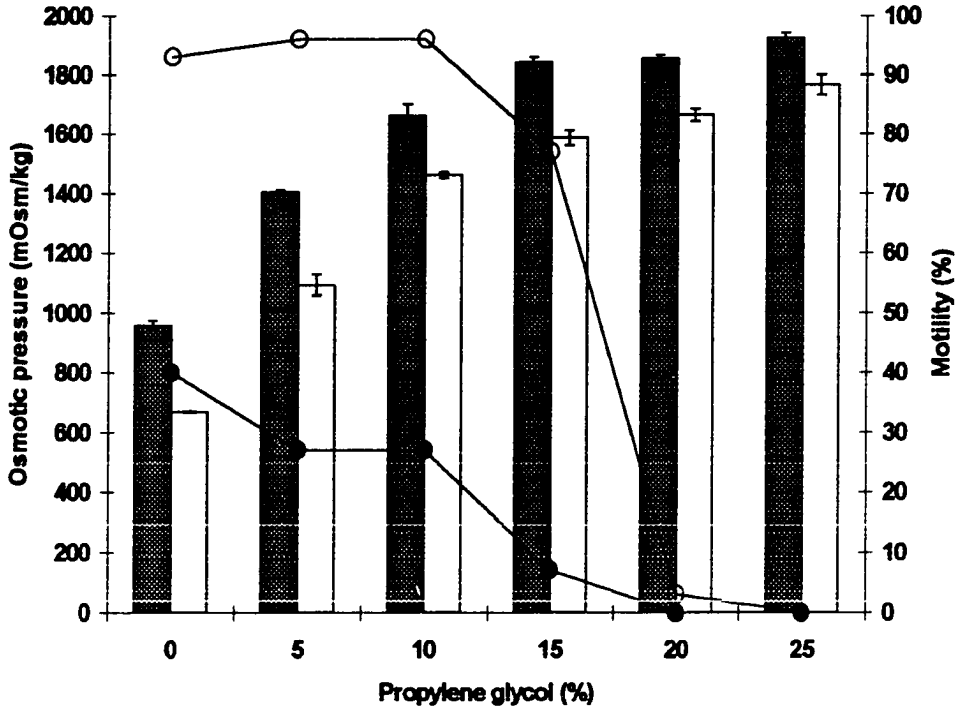


Figure 4.1 Relationship between osmotic pressure of the cryoprotectant solutions (CPS) and motility of sperm before cryopreservation. Open bars, CPS without addition of sucrose; shaded bars, CPS with sucrose; open circles, motility of sperm suspended in CPS without addition of sucrose; closed circles, motility of sperm suspended in CPS with sucrose.

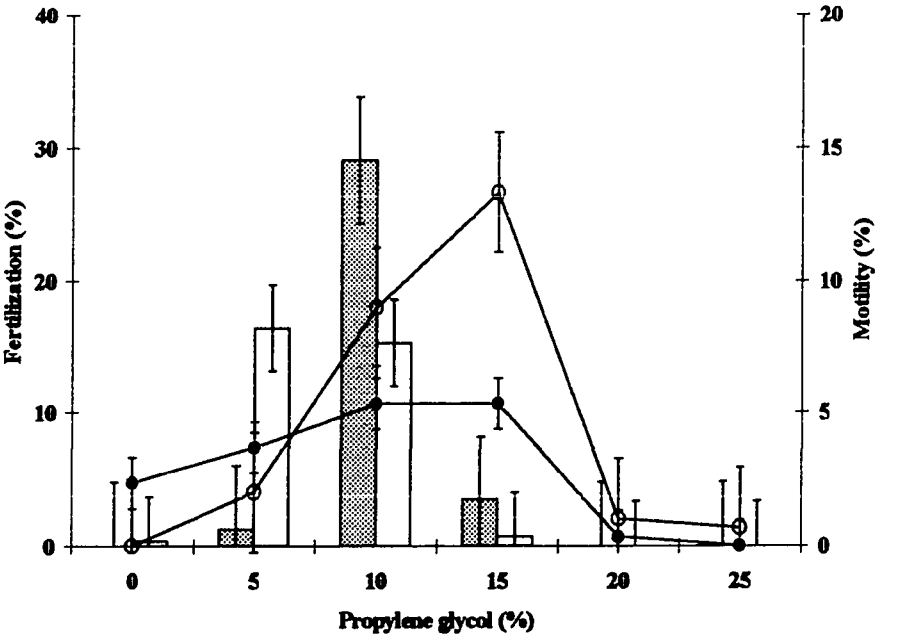


Figure 4.2 Motility and survival of trochophore larvae 12 h after fertilization with sperm thawed at 25 °C. Shaded bars, fertilization with sperm without sucrose; open bars, fertilization with sperm with sucrose; open circles, motility of sperm without sucrose; closed circles, sperm with sucrose.

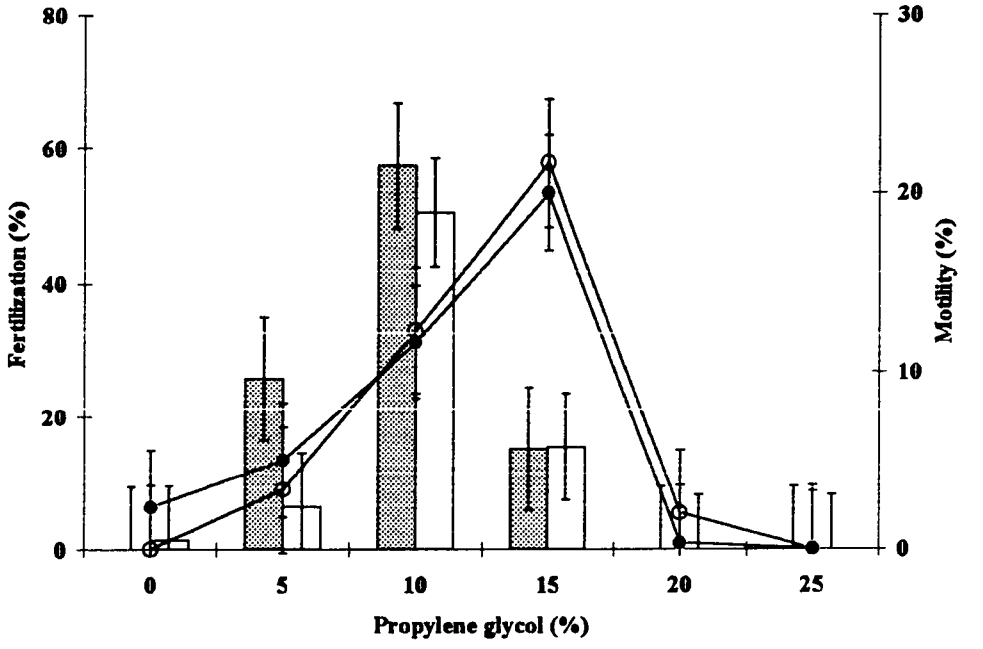


Figure 4.3 Motility and survival of trochophore larvae 12 h after fertilization with sperm thawed at 70°C. Shaded bars, fertilization with sperm without sucrose; open bars, fertilization with sperm with sucrose; open circles, motility of sperm without sucrose; closed circles, sperm with sucrose.

Table 4.4 Motility of eastern oyster sperm were analyzed using a three-way analysis of variance for the effect of cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%) presence or absence of sucrose (0.25M), and thawing temperature (25°C or 70°C). Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	P
Cryoprotectant	5	1.224	35.30	0.0001
Sucrose	1	0.000	0.02	0.9020
Thawing temperature	1	0.043	6.20	0.0163
Cryoprotectant x sucrose	5	0.101	2.90	0.0227
Cryoprotectant x thawing temperature	5	0.088	2.54	0.0405
Sucrose x thawing temperature	1	0.007	1.04	0.3126
Cryoprotectant x sucrose x thawing temperature	5	0.012	0.35	0.8809
Error	48	0.333		
Corrected total	71			

Variability of thawed sperm from individual males

We could not use the same eggs to assess the fertilizing ability of thawed and non-frozen sperm because the sperm was stored frozen for a month. A new batch of females was used and the fresh eggs were fertilized with pooled fresh sperm from five different males to test the egg quality. The fertilization of this batch was 90 $\pm$ 21% which indicated that the eggs were of good quality. The fertilizing ability of fresh sperm from individual males was not significantly different ($P = 0.8609$). Differences in fertilization were attributed to females ($P = 0.0001$) (Table 4.6). However, the fertilizing ability of thawed sperm from individual males was significantly different ($P = 0.006$) (Table 4.7).

Cryopreservation of trochophore larvae

Larval survival was affected by the concentration of larvae per straw ($P = 0.001$) (Table 4.8). The highest numbers of surviving larvae were found for a concentration of ~50,000 larvae per straw, although the highest percentage of survival was found for a concentration of 125 larvae per mL (Figure 4.6).

Table 4.5 Fertilizing ability of eastern oyster sperm was analyzed using a three-way analysis of variance for the effect of cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%) presence or absence of sucrose (0.25M), and thawing temperature (25°C or 70°C). Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	P
Cryoprotectant	5	4.097	12.99	0.0001
Sucrose	1	0.039	0.63	0.4314
Thawing temperature	1	0.406	6.44	0.0145
Cryoprotectant x sucrose	5	0.188	0.60	0.7035
Cryoprotectant x thawing temperature	5	0.683	2.16	0.0738
Sucrose x thawing temperature	1	0.063	1.00	0.3216
Cryoprotectant x sucrose x thawing temperature	5	0.199	0.63	0.6759
Error	48	3.029		
Corrected total	71			

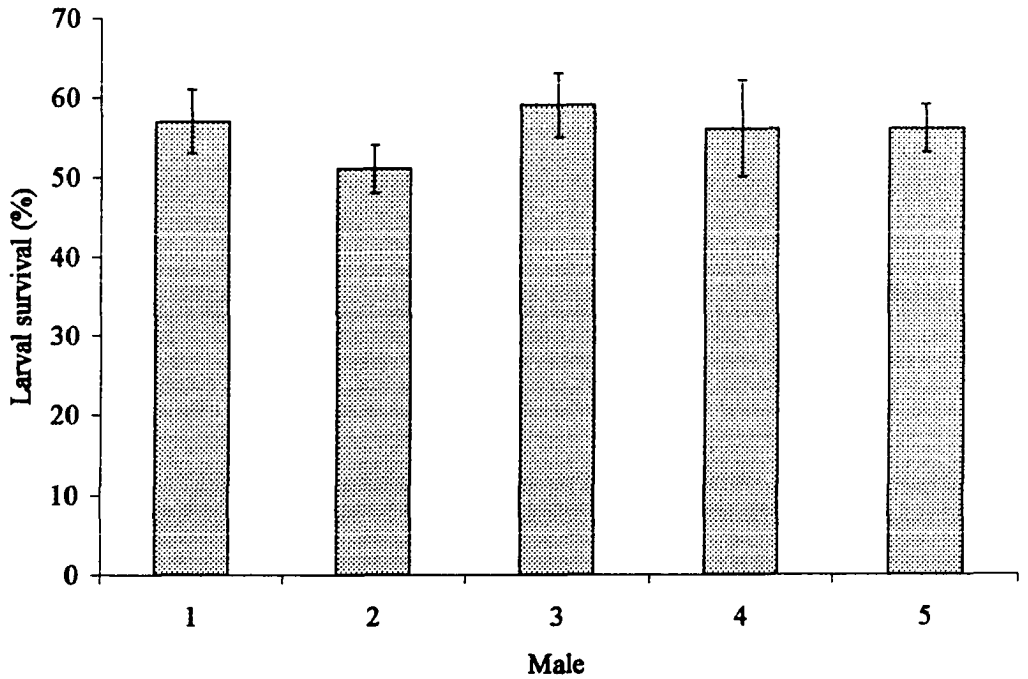
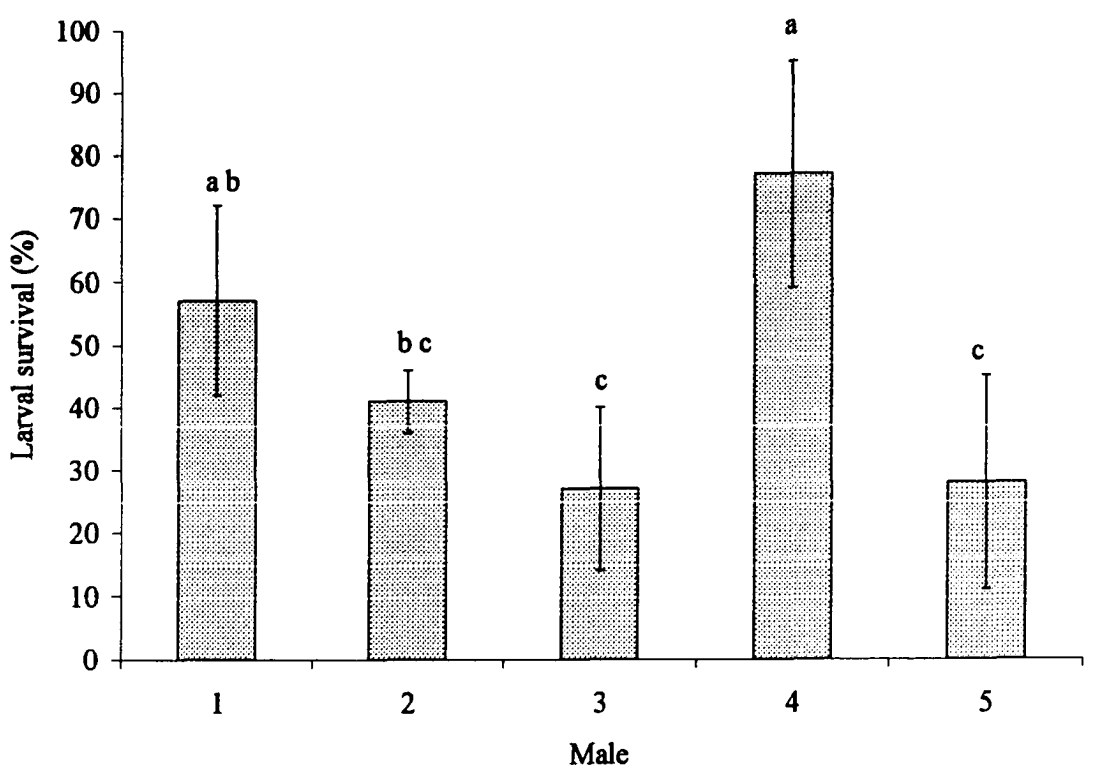


Figure 4.4 Fertilizing ability (mean ± SD) of eastern oyster sperm from five males before cryopreservation. There was no difference in fertilization among males.



Figures 4.5 Fertilizing ability (mean ± SD) of thawed eastern oyster sperm. Bars sharing letters were not significantly different.

Table 4.6 Variability of gamete samples from males and females before cryopreservation were analyzed using one-way analysis of variance. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	P
Male	4	0.033	0.32	0.8609
Female	4	0.331	32.43	0.0001
Error	16	0.041	--	--
Corrected total	24	--	--	--

Table 4.7 Variability of gamete samples from males and females after thawed sperm was used to fertilized fresh eggs were analyzed using one-way analysis of variance. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	P
Male	4	0.005	8.82	0.0006
Female	4	0.001	0.88	0.4962
Error	16	0.003	--	--
Corrected total	24	--	--	--

Table 4.8 Larval survival was analyzed using two-way analysis of variance for the effect of concentration of larvae per straw (125, 500, 5,000, 50,000,or 500,000) or cryoprotectant concentration (10% or 15% propylene glycol).

Source of variation	df	SS	F	P
Concentration	4	51541130	1575.22	0.0001
Cryoprotectant	1	3232080	395.12	0.0001
Concentration x cryoprotectant	4	19534870	597.03	0.0001
Error	10	81800	--	--
Corrected total	19	--	--	--

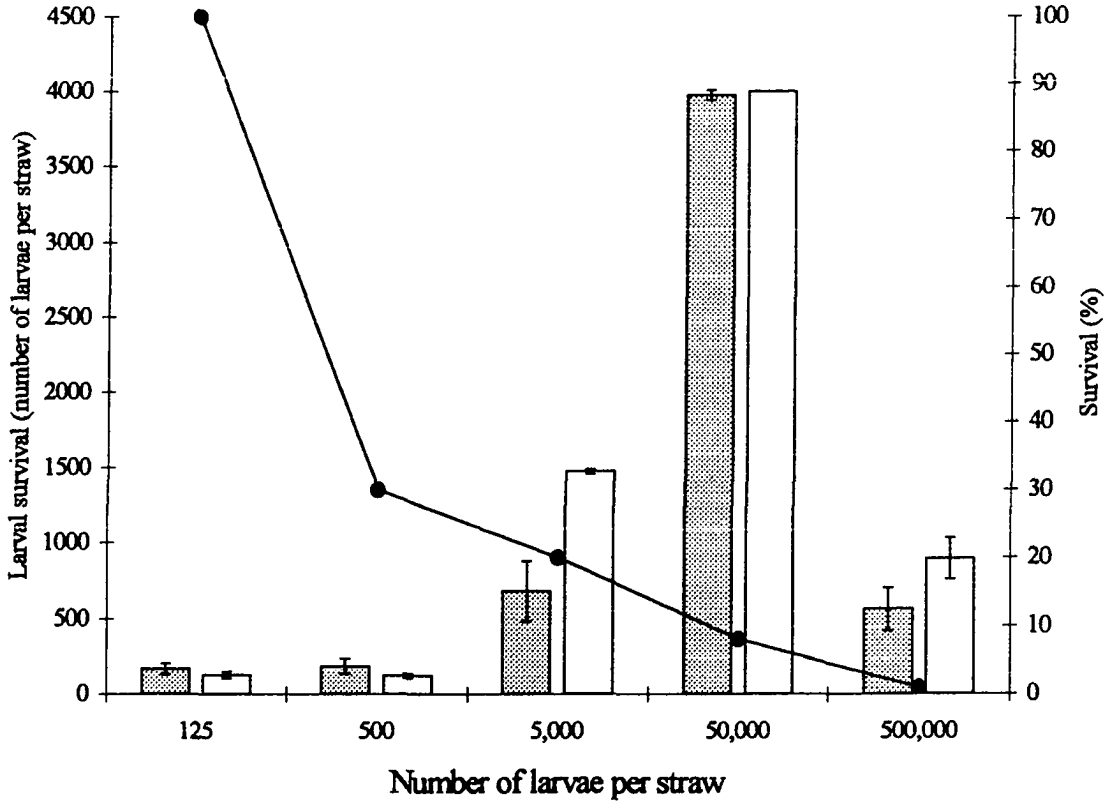


Figure 4.6 Number of surviving trochophore larvae after thawing and suspension in artificial seawater (ASW). Shaded bars, larvae cryopreserved in 10% propylene glycol; open bars, larvae cryopreserved in 15% propylene glycol. The line indicates average percent survival for each larval density.

DISCUSSION

In marine fishes, sperm are generally not motile in the testes and are activated only by release into the aquatic environment where the sperm are diluted in a media of high osmotic pressure (>400 mOsmol/kg) (Morisawa 1985). Motility duration of marine fish sperm is generally longer than that reported for freshwater fish (Billard 1988). Osmotic pressure of sperm solutions or cryoprotectant solutions is of interest because it can be an important factor in the cryopreservation process. In mice, for example, it has been reported that there are different osmotic tolerances among strains and that the damage caused by osmotic stress occurred mostly after exposure of the sperm to the freezing medium and dilution into isotonic medium (Songsasen and Leibo 1997).

The first study on cryopreservation of eastern oyster sperm reported that eggs fertilized with thawed sperm (35 min after insemination) yielded fertilization of 2% (Hughes 1973). A later study reported growth of D-stage larvae from naturally spawned eggs fertilized with thawed sperm of the eastern oyster (Zell et al.). However, the experimental conditions in that study were difficult to reproduce. The samples were intended to be frozen at –5°C per min, but the actual cooling rates were erratic. Another problem was that in some trials sperm samples were combined. Thus, the fertilizing ability of thawed sperm from individual males could not be tested.

In this study, it was found that when sperm were suspended in C-F HBSS plus sucrose and no cryoprotectant (958 mOsmol/kg), the motility decreased from 90% to 35%. The sperm regained motility when they were suspended in C-F HBSS without sucrose. However, it could not be concluded that an increase in the osmotic pressure was responsible for the decrease of motility because sperm suspended in 5% and 10% propylene glycol reached osmolalities higher than 1000 mOsmol/kg and did not lose motility.

The highest fertilization was found with 10% propylene glycol, although the highest motility was found at 15% propylene glycol. This indicates that motility was not a direct indicator of fertilizing ability and that other techniques such as fluorescent microscopy or flow cytometry will be required to evaluate the quality of thawed sperm. Inconsistency in the quality of sperm after thawing has been discussed in several cryopreservation studies (Baynes and Scott 1987). Motility is a routine method to estimate fertilizing ability of fresh sperm, but motility estimation does not always correlate with fertilization rates after thawing (Stoss and Holtz 1983; Piironen 1987). This inconsistency has been attributed partly to biological variation such as aging (Baynes and Scott 1987). In the present study,

there was no difference in fertilizing ability of fresh sperm from five males; however, the fertilizing ability of thawed sperm varied among males. It is possible that sperm of different males vary in their response to freezing and thawing. More studies need to be performed to investigate variation in fertilizing ability of thawed sperm.

For larvae, techniques previously reported for the Pacific oyster (Gwo 1995) were adapted. However, this previous report did not identify thawing rates, concentration of larvae per straw or type of straw used. These factors were important for experiments at the hatchery level. For practical purposes, 5-mL macrotubes were used to store large numbers of larvae (thousands or millions per macrotube). It was found that larval survival was affected by larval concentration. Macrotubes containing few larvae (125 larvae per mL) yielded complete survival after thawing. However, straws containing 50,000 larvae per straws yielded the highest numbers of larvae >4,000 per straw. Another important factor was the quality of larvae. We found differences in the quality of larvae from batch to batch, and vigorous larvae were less affected when stored at concentrations of higher than 5,000 larvae per macrotube.

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CHAPTER 5
HATCHERY PRODUCTION OF EASTERN OYSTERS FROM CRYOPRESERVED LARVAE AND SPERM

In aquaculture, cryopreservation studies have primarily addressed spermatozoa of commercially important fishes (salmonids, tilapias and carps) (Stoss 1983). However, work with larvae of fish or other organisms such as mollusks is scarce (Gwo 1995). In mollusks, the Japanese oyster Crassostrea gigas has been the species most used to study cryopreservation of larvae (Table 5.1). Other species of oysters, including those of commercial importance, have not been studied. In addition, cryopreservation of oyster gametes and larvae has, with only a single exception, been tested in the laboratory, and has yielded a single report of oyster growth beyond planktonic stages (Paniagua et al. 1998). Given the benefit that this technique offers to research and the commercial oyster industry, cryopreservation of oyster gametes and larvae should be developed for application in the hatchery.

Production of eastern oysters along the Atlantic and Gulf coasts of the United States has declined due to a lack of consistent seed supply, excessive harvest, disease and natural predation (Supan and Wilson 1993). The production of cryopreserved gametes or larvae would improve hatchery production of seedstock oysters, allow distribution of improved or genetically modified stocks, and provide feed for larval marine fish. This study represents the first successful production of seedstock from cryopreserved larvae of any aquatic food organism cultured for human consumption (Paniagua et al. 1998) and it documents for the first time the settlement and growth of seed oysters produced from eggs fertilized with thawed sperm.

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Table 5.1 Previous studies of cryopreservation of gametes and larvae of oyster species.

Species	Material frozen	Citation
C. virginica	Sperm	Huges 1973
C. virginica	Sperm	Zell et al. 1979
C. gigas	Sperm	Bougrier and Rabenomanana 1986
C. tulipa, C. iredalei, S. cucullata and C. gigas	Sperm	Yankson and Moyse 1991
C. gigas	Sperm	McFadzen 1995
C. gigas	Larvae	Renard and Cochanrd 1989
C. gigas	Larvae	Renard 1991
C. gigas	Larvae	McFadzen 1993
C. gigas	Larvae	Lin et al. 1993
C. gigas	Larvae	Chao et al. 1994
C. gigas	Larvae	Lin et al. 1994
C. gigas	Larvae	Gwo 1995
C. gigas	Larvae	Chao et al. 1997
C. gigas	Larvae and eggs	Naidenko 1997

MATERIALS AND METHODS

Gamete Quality

Collection of high quality gametes was necessary for reliable production of oyster larvae suitable for cryopreservation. Eastern oyster can tolerate a wide range of salinities (osmolalities) in the natural environment. In the laboratory, osmolality must be controlled for gametes and larvae to be used for cryopreservation. Because osmolality can affect gamete quality (Figure 5.1), oysters from natural environments were held in recirculating systems in the laboratory for at least 5 days at 18 to 20°C. This temperature inhibited Gulf Coast oysters from spawning, but allowed maintenance of ripe gametes when the oysters were fed the marine microalgae Isochrysis galbana (T-iso) and Chaetoceros calcitrans. This acclimation period also allowed oysters that were stressed or weakened in transport to recover or die (reducing bacterial contamination of gametes and larvae). A marked increase in production of larvae was noted after acclimation (Figure 5.2), and in some cases larvae could not be produced until after 5 days of acclimation. Oysters were held for as long as several months at 18 to 20°C, inhibiting spawning while allowing gamete maturation and retention (Buchanan et al. 1999).

Before gamete collection, oysters were scrubbed with a mild bleach solution (5%) or were washed with 70% ethanol. Oysters were opened and visually inspected for ripeness indicated by a creamy color with prominent genital canals running throughout the gonad. Mature oysters were selected and rinsed with 70% ethanol, followed by rinsing with artificial seawater (ASW). To determine sex of the oysters, a sample from each gonad was taken by puncture with a capillary tube.

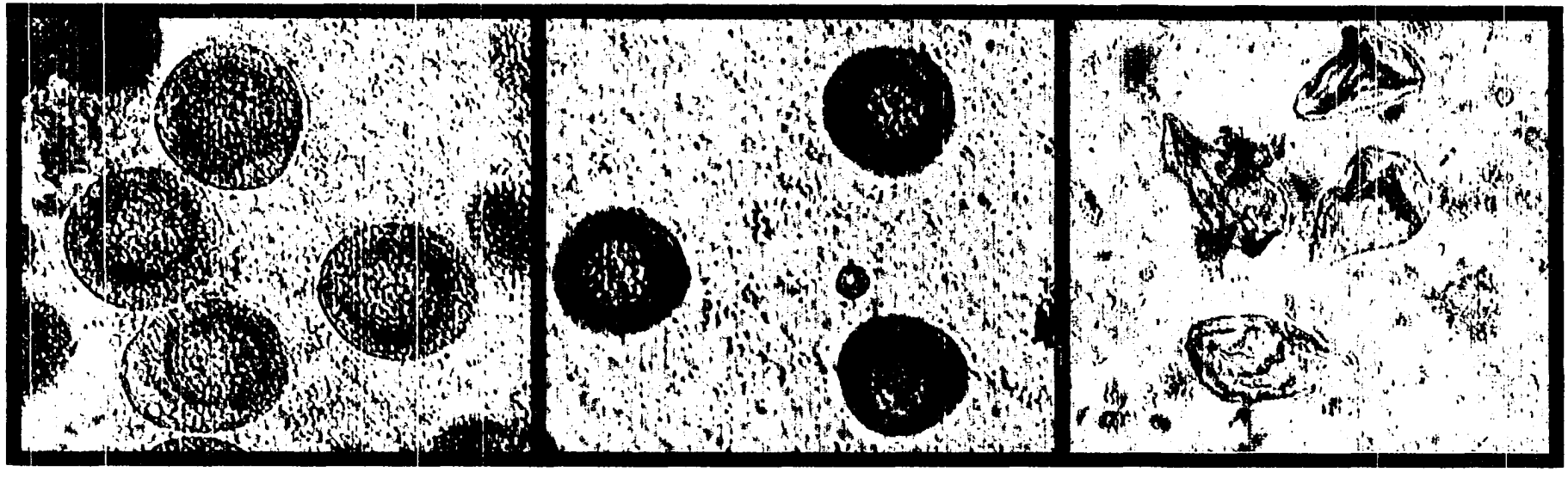

Figure 5.1 Eggs of the eastern oyster exposed for 5 min to artificial seawater (ASW) of different osmolalities. Prior to exposure, eggs were held at laboratory osmolalities (475 mOsm/kg) for 5 days. Left panel, eggs placed in hypotonic ASW at 115 mOsmol/kg; middle panel, eggs placed in isotonic ASW at 468 mOsmol/kg; right panel, eggs placed in hypertonic ASW at 705 mOsmol/kg.

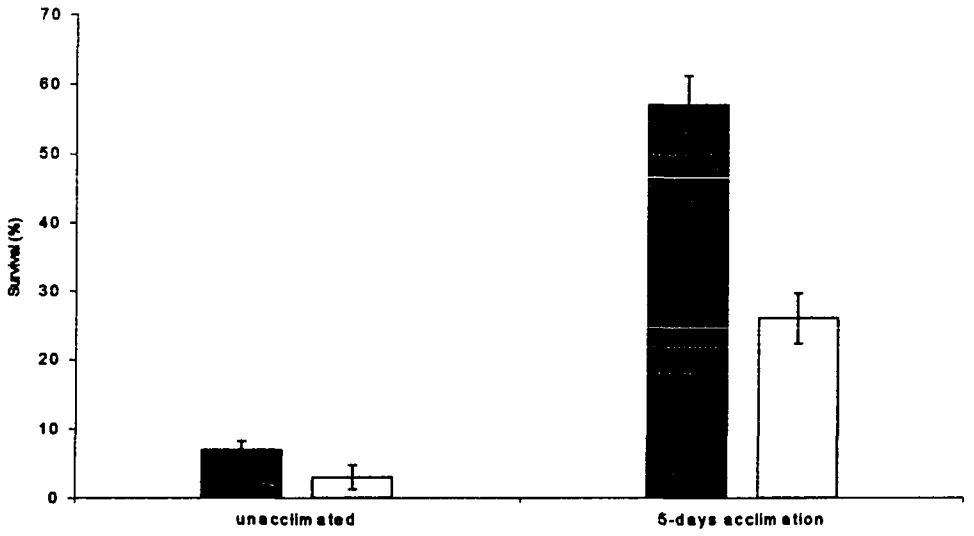

Figure 5.2 Larval survival (mean ± SD) of eastern oysters at 12 h (black bars) and 24h (open bars) after fertilization. Oysters were selected at random for spawning before and after laboratory acclimation (5 days). Percent survival was the average for eggs of four females.

This sample was smeared onto a glass slide and observed at 100X magnification with a phase-contrast microscope. The gonads were lacerated with a sterile scalpel and the gametes were removed with a glass Pasteur pipette and placed into clean plastic beakers. All egg samples were passed through a 70-μ Nitex screen and were collected on a 13-μ screen. Eggs were washed with ASW and collected in a clean beaker. For sperm, samples were washed with C-F HBSS (Paniagua et al. 1998) through 70-μ and 13-μ screens, and were suspended in C-F HBSS. Only actively swimming sperm were considered to be motile, and only males with sperm motility greater than 90% were selected for experimentation. To produce larvae, eggs were mixed with ~500 sperm per egg in 1 L of ASW yielding a concentration of ~100 embryos per mL. Trochophore larvae were collected ~12 h after fertilization (incubated at room temperature, 21°C) on a 13-μ screen. Osmolality of all solutions was 475 mOsmol/kg. A 0.45-μ filter was used to sterilize the C-F HBSS, and ASW was passed through a 1-μ filter, sterilized with ultraviolet irradiation, and passed through an activated carbon filter before use.

Cryopreservation protocol

A single protocol was used to cryopreserve sperm and larvae (described below). Trochophore larvae were frozen at a concentration of 10,000 per mL, and sperm were frozen at ~1 x 10^9 per mL. After suspension of sperm in C-F HBSS or trochophore larvae in ASW, they were placed in a cryoprotectant solution composed of ASW and a final concentration of 15% propylene glycol. Five-mL macrotubes (Minitube of America, Inc., Madison, Wisconsin) were filled with the sperm or larvae suspensions and allowed to equilibrate for 20 min at 21°C. The macrotubes were frozen in a controlled-rate freezer (Kryo 10 series II, Planer, England). The cooling rate was –2.5 °C per min until reaching a final temperature of –30 °C which was held for 5 min. Macrotubes were plunged into liquid nitrogen and stored for one week. A water bath was used to thaw the samples at 70°C for 15 sec.

Hatchery experiment

Samples were transported to a hatchery at Grand Isle, Louisiana, in nitrogen vapor shipping dewars (Taylor Wharton, model CP-65, Theodore, Alabama). In the hatchery, the macrotubes were thawed in a water bath at 70°C for 15 sec and drained into 1-L plastic beakers containing an equal volume of fresh filtered seawater. Thawed sperm were used to fertilize freshly stripped eggs. A 30-μ mesh screen was used to remove thawed larvae from the water with cryoprotectant. Larvae collected on the screen were ready for culture in tanks.

RESULTS

The experiments were performed in two consecutive years starting on September 18, 1996 and September 20, 1997 (Table 5.2). In the first year, the experiment was terminated after 10 days due to an approaching hurricane and flooding at the site (Grand Isle,

Louisiana). After 24 h of incubation, 10% of the control larvae had developed to D-stage. Subsequently, survival of the control larvae decreased rapidly, and after 10 days larvae were not found. Two percent of thawed larvae developed to D-stage and after 10 days of incubation 1,000 pediveligers were counted. Eggs fertilized with thawed sperm yielded better survival. After 24 h, 1.1×10^6 D-stage larvae were counted and after 10 days of incubation 2.4×10^4 pediveligers were found.

In the second year, thawed larvae were incubated in a 200-L tank for 10 days, fed daily with 20 L of the algae I. galbana and treated as a normal hatchery brood. After 24 h of incubation, 28% of the control larvae, 24% of thawed larvae, and 88% of larvae produced with thawed sperm had developed to D-stage. Larval numbers decreased throughout development, indicating absence of contamination with wild larvae. Larvae were collected by screening daily on 210-μ mesh beginning after 8 days of incubation, and were transferred to a system containing cleaned shells of the common clam Rangia cuneata used as cultch material. A total of 500,000 control larvae, 28,000 thawed larvae, and 64,000 larvae produced with thawed sperm were placed in the cultch system. After settlement, spat were secured in plastic mesh bags and suspended in Caminada Bay (29° 15' 12" N, 90° 03' 26" W) to evaluate survival and growth. After 2 weeks of suspension in the Bay, a subsample of 80 Rangia shells were obtained at random from each bag to count spat. One thousand spat were counted from the control group, 200 spat from the thawed larvae, 80 from larvae produced with thawed sperm, and none from clean shells available for settlement of wild spat.

Table 5.2 Number of eastern oyster produced from control and thawed larvae and larvae produced with thawed sperm during 2 years of research.

1996					
Date	Control larvae	Thawed larvae	Survival (% control)	Thawed sperm	Survival (% control)
9/18	3.0×10^6	3.0×10^6	100%	3.0×10^6	100%
9/20	3.1×10^5	5.0×10^3	2%	1.1×10^6	355%
9/22	3.8×10^4	3.6×10^3	9%	2.8×10^5	737%
9/24	6.0×10^3	2.0×10^3	33%	7.9×10^4	1,317%
9/26	4.6×10^3	2.0×10^3	43%	3.4×10^4	739%
9/30*	0	1.0×10^3	--	2.4×10^4	--

1997					
Date	Control larvae	Thawed larvae	Survival (% control)	Thawed sperm	Survival (% control)
9/20	6.0×10^6	3.0×10^6	50%	3.0×10^6	50%
9/22	1.7×10^6	4.0×10^5	24%	1.5×10^6	88%
9/24	1.0×10^6	2.0×10^5	20%	6.3×10^5	63%
9/26	1.0×10^6	5.3×10^4	5%	5.0×10^5	50%
9/28	5.6×10^5	2.8×10^4	5%	6.4×10^4	11%
Set on cultch					
10/97**	1.0×10^3	2.0×10^2	20%	8.0×10^2	8%
1/98***	1.0×10^3	8.5×10^2	85%	2.3×10^2	23%

*Experiment terminated due to approaching hurricane.
**Total number of spat counted on a subsample of 80 shells of Rangia cuneata (cultch). No natural spatfall was observed on any cultch samples on this date.
***Natural spatfall was observed only on clean cultch (not in control or thawed treatments). Oysters from wild spatfall were visibly smaller than the experimental oysters.

After 4 months of suspension in the Bay, all of the cultch material was examined and a total of 1,000 seed oysters (2.5 to 5 cm) were counted from the control group, 850 were counted from the thawed larvae and 230 from larvae produced with thawed sperm. At this time, a total of 57 wild spat were collected from the bags containing clean cultch (23 on one bag and 34 on the other). This was the equivalent of about 30 wild spat per treatment (bag). The wild spat were less than 0.5 to 1 cm in length, while the experimental spat (from control and thawed larvae) were more than 2.5 cm at this time, allowing identification of naturally produced spat.

The combination of few natural spat at this time of year and a greater suitability for settlement on the clean cultch may explain the lack of wild spat in the experimental bags. During the experiments all larvae and spat appeared to be morphologically normal and healthy (Figure 5.3).

Experimental safeguards

Working at a hatchery on the coast created benefits and problems. We benefited from the availability of natural seawater, ambient conditions and natural (heterogeneous) food sources for growth of oysters after settlement. On the other hand, we needed to ensure that no extraneous natural larvae contaminated the experiment before settlement, and that natural spatfall did not influence our results.

Before settlement, we used several approaches to exclude contamination by extraneous larvae. We chose to work during late September which is at the end of the spawning season in Louisiana, thereby reducing the numbers of natural larvae in the waters near the hatchery. All oyster and algae tanks were segregated from sources of natural water and other sources of contamination. All equipment and mesh screens were used exclusively for this experiment and were washed with filtered water before and after use. All water

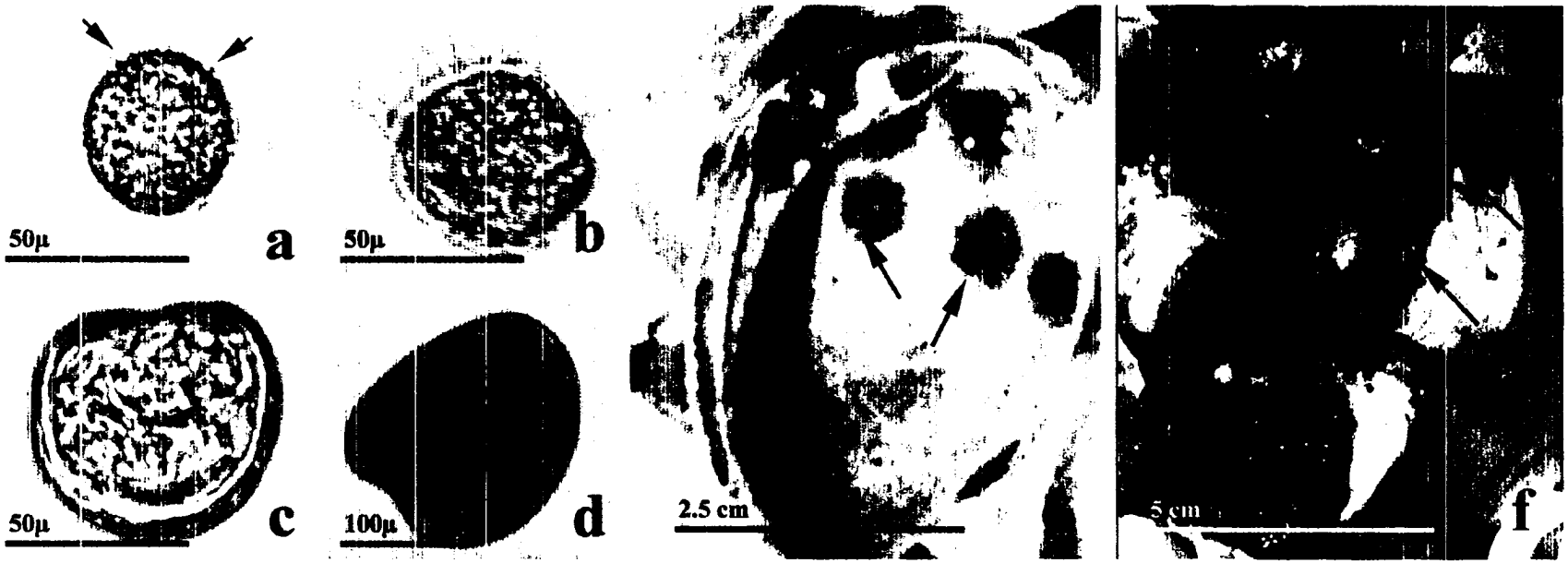

Figure 5.3 Development of the eastern oyster Crassostrea virginica. **a:** Fertilized egg, arrows indicate sperm surrounding the egg; **b:** trochophore larvae; **c:** D-stage larvae; **d:** pediveliger (formalin-fixed); **e:** spat, arrows indicate spat 2 weeks after settlement on shell of Rangia clam; **f:** seed oysters, arrows indicate seed oysters 4 months after settement.

used for rearing of oyster larvae and for culture of algae was filtered repeatedly through mesh sizes down to 1 μ to prevent contamination. The embryo and larvae in the experiments were collected on appropriate-sized mesh screens every 2 days to perform water exchanges and counts. This eliminated contamination of experimental larvae by earlier (smaller) stages of natural larvae. Experimental larvae were counted at each handling, allowing us to note any increases in numbers due to contamination (Table 5.2). The larvae were also monitored to ensure that the populations were of the correct developmental stage. As a final check of the larvae, we performed counts of eyed pediveligers as they were collected to be moved to the tanks containing cultch. The number collected for setting was compared with the larval census at that developmental stage.

After settlement, spat were placed in mesh bags and suspended in the waters of Caminada Bay for future growth. Because some amount of natural spat settlement was unavoidable, we suspended two additional mesh bags containing clean cultch among the experimental bags to estimate the occurrence of natural spat fall. The oysters were inspected at 2 weeks and at 2-month intervals thereafter. Relative size was used to differentiate between experimental and wild spat.

DISCUSSION

The objective of applied research is the transfer of technology from the laboratory to the industrial or commercial level. In aquaculture, technology transfer involves modification of laboratory techniques for use in hatcheries or other culture environments. Cryopreservation of gametes and embryos is a good example of technology that can be used in aquaculture. However, most of the studies on cryopreservation of oyster sperm and embryos have been limited to a few days in the laboratory and have not been

evaluated for production. It is important to evaluate laboratory studies because often they do not yield the same results as those performed in the hatchery or natural environment. The growth of thawed larvae and larvae produced from eggs fertilized with thawed sperm for a few hours or days after thawing does not ensure survival through settlement or production of adult oysters. Genetic or physiological damage could result in production of abnormal larvae, excessive mortality, or the inability to complete metamorphosis and attachment to cultch.

In this study, it was possible to obtain settlement and growth of normal seed oysters produced from thawed trochophore larvae and eggs fertilized with thawed sperm. Survival although apparently low, was not different from the control group, and was not different than normal survival in the hatchery. Extremely large numbers of larvae (in the billions) are routinely used to produce seedstock at the commercial level. With additional research, cryopreservation could be scaled up to allow production of commercially relevant numbers of larvae.

This is the first successful production of seedstock from cryopreserved larvae of any species of aquatic organism commercially grown for human consumption. This is also the first production of seed oyster from eggs fertilized with thawed sperm. The production of oysters capable of being planted on oyster grounds for growth and harvest demonstrates that cryopreservation can be applied for use in the hatchery. This is especially important for mollusks because, at present, maintenance of broodstock requires considerable space and expense within a hatchery, or entails the risk of placing valuable stocks into natural waters where they are susceptible to diseases, predators and contamination with wild spat fall.

The availability of frozen larvae and sperm offers obvious benefits. The frozen material would ensure protection of valuable stocks and would facilitate transport and availability of improved (e.g. disease-resistant) lines. The management of genetic resources of endangered species (Tiersch et al. 1998), and the techniques for larval cryopreservation may be applicable to highly endangered species such as the freshwater mussels (family Unionidae) which are the most rapidly disappearing fauna in North America (Williams et al. 1993). Research efforts would benefit from standardization (over distance and time) available through cryopreservation of control lines and economical storage of specific research populations (e.g. tetraploid larvae). Work could proceed year round given the availability of larvae outside of the spawning season. This point has special relevance to cytogenetic studies of oysters given that transformed cell lines do not exist, and larval material is often used to yield primary cultures with sufficient mitotic activity to reliably produce metaphase chromosomes (Zhang et al. 1999). However, future studies should evaluate the complete life cycle of oysters produced from cryopreserved larvae and sperm, and address optimization of production.

A final point to be considered is the economic feasibility of the cryopreservation of oyster sperm and larvae. A cost analysis has been performed for cryopreservation of fish sperm (Caffey and Tiersch 2000). Given the possibility of using cryopreserved oyster larvae and sperm in the hatchery, a similar analysis should be performed for a scaling-up of production.

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CHAPTER 6
ASSESSMENT OF THE QUALITY OF OYSTER GAMETES

In most cases, sperm motility has been the most used method to assess viability of thawed sperm despite variable correlation to fertility (Stoss and Holtz 1983; Soderquist et al. 1991). Freezing and thawing of sperm can lead to severe impairment of cellular function resulting in reduced fertility (Hammerstedt, et al. 1990). Sperm cells consist of several membrane-bound compartments such as those bounded by the plasma membrane, acrosomal membrane and mitochondrial membrane and cell competency requires that each of these membranes and compartments remain intact (Graham et al. 1990).

In oysters, only one study has been performed to evaluate the factors that decrease fertilizing ability of thawed sperm (Kurokura et al. 1990). Observations of survival, motility and morphology of thawed sperm of the Pacific oyster, Crassostrea gigas, were performed using light microscopy and scanning electron microscopy (SEM). The use of SEM can be useful, but it is time consuming and labor intensive. Newer technologies have been developed such as flow cytometry which can provide an estimation of viable sperm cells, and with proper protocols, can be rapid and relatively simple to perform (Mc Niven, et al., 1992). Simultaneous evaluation of two or more properties of sperm by use of dual-staining techniques have been used with success in other species such as turkeys (Donoghue et al., 1995) and bulls (Evenson et al. 1993; Ericson et al. 1993) and could provide definitive information about the functional status of oyster sperm.

Sperm viability is one of the properties that has been determined using dual-staining with the fluorescent dyes Sybr-14 and propidium iodide (Garner et al. 1994). Sybr-14 is able to penetrate the sperm head and stain the nucleic acids of viable cells. The other dye, propidium iodide (PI) is used to identify non-viable cells because it can only

penetrate damaged nuclear membranes and stain nucleic acids by intercalating between the base pairs.

A mixture of two fluorescent dyes, rhodamine 123 (R 123) and PI can be used to evaluate the mitochondrial function of sperm cells. Rhodamine 123 is a cationic, fluorescent dye widely used as an indicator of oxidation potential. It provides high-resolution fluorescent images of mitochondria with little background and no apparent cytotoxic effects (Johnson et al. 1980).

These dyes can be analyzed by use of a flow cytometer; however, the analysis of oyster eggs by flow cytometry presents problems. One of the principal reasons for this is that lipid droplets from broken eggs can disrupt sample flow through the instrument. The use of fluorescent microscopy to evaluate staining procedures may be an option for the analysis of oyster eggs. Vital stains can be used to distinguish between viable and non-viable cells. Neutral red is useful to stain viable eggs, although, if the stained eggs are fertilized, the dye can be toxic to developing embryos (Kardymowicz 1972). Fluorescein diacetate is one of the most suitable dyes used to test the viability of plant cell cultures (Widholm 1972). The non-fluorescent, non-polar dye, fluorescein diacetate (FDA), can penetrate cells and be hydrolyzed by intracellular esterases to the polar, fluorescent compound fluorescein which accumulates in intact cells. Fluorescein is released though damaged cell membranes and therefore intracellular accumulation of the compound can be used as a indicator of membrane integrity (Boender 1984).

Cryopreservation of eggs of the eastern oyster, C. virginica, has not been performed. A first step in cryopreservation would be to evaluate possible toxic effects of cryoprotectants on the viability of eggs. One method could be to follow the development of fertilized eggs previously treated with cryoprotectant. Another method could be the

use of vital dyes. Fluorescein diacetate may be useful to determine the viability of eastern oyster eggs before and after treatment with cryoprotectants and after thawing.

The objectives of this work were to: (1) develop flow cytometric techniques to assess sperm viability; (2) assess the viability and mitochondrial function of thawed eastern oyster sperm by use of flow cytometry, and (3) assess the viability of eastern oyster eggs by use of fluorescein diacetate before and after addition of cryoprotectant and after thawing.

MATERIALS AND METHODS

Flow cytometric techniques

(1) Standard curves. To produce non-viable cells used to generate a standard curve for known ratios of viable and non-viable sperm, 10-mL samples of diluted sperm from three males (final concentration of 1 x 10^8 cells/mL) were exposed to the following treatments: (a) 1-mL samples were placed in a freezer (-20°C) for 15 min. The samples were thawed at room temperature (21°C) and this was repeated 3 times; (b) 1-mL samples were plunged directly in liquid nitrogen for 15 min and thawed at room temperature; (c) 1-mL samples were exposed to 50°C or 65°C for 10 min and cooled to room temperature or; (d) 1-mL samples were exposed to 10% methanol for 15 min. After each treatment, the samples were evaluated by light microscopy at 200 X. (Table 6.1.)

Table 6.1 Treatments used to produce non-viable eastern oyster sperm.

Treatment	Result	Motility (%)
Cooling to –20°C	Motile sperm	1
Plunging in liquid nitrogen (-196°C)	Agglutination of sperm	0
Heat (>60°C)	Agglutination of sperm	0
Heat (50°C)	Non-motile sperm	0
10% methanol	Agglutination of sperm	0

Based on these experiments, heating to 50°C was chosen to produce non-viable sperm. The exact physiological status of these heat-treated sperm is difficult to determine, but they will be referred to as being "non-viable" in comparison to the untreated fresh sperm which will be referred to as being "viable." Viable and non-viable sperm were mixed in five ratios (100:0, 75:25, 50:50, 25:75 or 0:100) and were stained with the fluorescent dyes (Molecular Probes, Eugene, Oregon) Sybr-14 and PI to determine viability, or with R123 and PI to evaluate mitochondrial function. To optimize dye concentrations, sperm were stained at several concentrations (Table 6.2). A Sybr-14 solution stock was prepared that contained 1 mM of dye in 100 μL of DMSO. For the experiment, a 1:50 dilution in DMSO was prepared from the stock solution resulting in a final concentration of 20 nM. A PI solution stock was prepared that contained 1.6 g (2.4 mM) per mL of water. A R123 solution stock was prepared that contained 0.01 g (2.6×10^{-5} mM) of dye per mL of water. All stock solutions were stored in the freezer at 20°C.

Table 6.2 Concentrations of dyes used to stain viable and non-viable sperm

Rhodamine 123	Sybr-14	Propidium iodide
100 nM	100 nM	24 μM
0.07 nM	50 nM	12 μM
0.03 nM	20 nM	6 μM

No differences in assay results were found among the different concentrations of dyes. The concentrations chosen for further analyses were: 0.03 nM for R123, 20 nM for Sybr-14 and 12 μM for PI.

The samples were analyzed using a flow cytometer (FACSCalibur® Model, Becton Dickinson, San Jose, California) equipped with an air-cooled 480-nm argon (blue) laser. The FACScomp® software provided by the manufacturer (Becton Dickinson) was used to standardize equipment settings. To estimate sperm viability, 5μL solution stock of Sybr-14 was added to a 1-mL sample, incubated for 10 min, and 5μL of solution stock of PI were added. To estimate mitochondrial function, 1μL of solution stock of R123 was added to 1-mL sample, incubated for 10 min, and 5μL of solution stock of PI was added. All samples were incubated for 10 min prior to analysis. A total of 10,000 sperm cells were analyzed per sample. Quantitative data on fluorescent-stained populations were collected using forward-scattered and side-scattered light. To collect viability data, density plots using green fluorescence for viable sperm (Sybr-14) and red fluorescence for non-viable sperm (PI) were used. To estimate mitochondria function, density plots using green fluorescence for functional mitochondria (R123) and red fluorescence for non-viable sperm (PI) were used. Data were analyzed using the Cell Quest Software (Becton, Dickinson). Data analysis obtained from the flow cytometer involves the use of gates (areas designated specifically for viable or non-viable sperm) to determine the percent of viable sperm in a population. Sperm gated in the green fluorescent area were considered to be viable if stained with Sybr-14, and to have functional mitochondria if stained with R123. Sperm gated in the red fluorescent area were considered to be non-viable (Fig 6.1)

From the results of this experiment, two standard curves were generated, one for sperm viability, and one for mitochondrial function. A simple linear regression was used to determine the correlation between the predicted and measured ratios of viable and non-viable sperm.

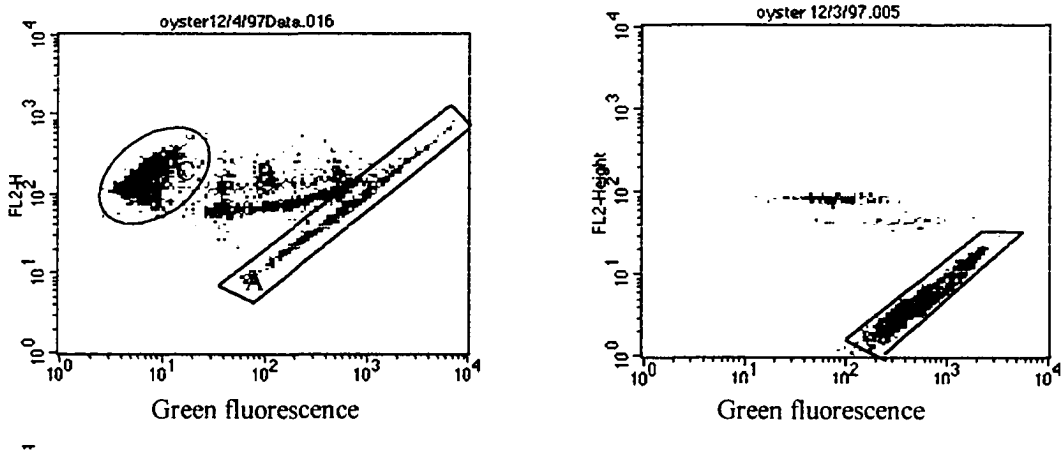

Figure 6.1 Flow cytometric analysis of thawed eastern oyster sperm. Left panel, thawed sperm stained with Sybr-14 and propidium iodine; right panel, thawed sperm stained with rhodamine 123 and propidium iodide. A, viable sperm; B, transitional sperm; C, non-viable sperm.

Viability and mitochondrial function of thawed eastern oyster sperm

Samples of cryopreserved sperm were analyzed using flow cytometry. Sperm samples from three males cryopreserved in six different concentrations (0%, 5%, 10%, 15%, 20% and 25%) of the cryoprotectant propylene glycol (PG) were thawed at 25°C or 70°C and were diluted in calcium-free Hanks' balanced salt solution (C-F HBSS). Aliquots of 20 µL of sperm were suspended in 1 mL of C-F HBSS (final concentration, ~2 x 10^6 sperm per mL). Samples were prepared as described above to estimate sperm viability and mitochondrial function.

Data analysis

A multiple linear regression analysis was used to relate motility and fertilizing ability of thawed sperm with the flow cytometric results, cryoprotectant concentrations, thawing temperature, and males. Statistical analyses were performed using SAS software for Windows® (SAS Institute, Cary, North Carolina).

Effect of cryoprotectant solutions on the viability of eastern oyster eggs

Eggs from ripe females were obtained and treated as described in Appendix A. Eggs from three females were pooled and ~4,000 eggs were placed for 5 min in each of the cryoprotectants listed in Table 6.3. After incubation, the eggs were washed in fresh-filtered artificial seawater (ASW) of 519 mOsmol/kg for 5 min.

Table 6.3 Cryoprotectant solutions used for cryopreservation of eastern oyster eggs.

Cryoprotectant (Molarity)
Dimethyl sulfoxide (0.50 M)
Dimethyl sulfoxide (0.86 M) and sucrose (0.14 M)
Dimethyl sulfoxide (2.00 M)
Dimethyl sulfoxide (1.75 M) and sucrose (0.25 M)
Dimethyl sulfoxide (2.63 M) and sucrose (0.38)M
Dimethyl sulfoxide (3.50 M) and sucrose (0.50 M)
Propylene glycol (0.86 M) and sucrose (0.14 M)
Propylene glycol (1.75 M) and sucrose (0.25 M)
Propylene glycol (2.63 M) and sucrose (0.38 M)
Propylene glycol (3.50 M) and sucrose (0.50 M)

Eggs were suspended in ASW at a concentration of 35 eggs per mL and were fertilized with fresh sperm. Fertilized eggs were incubated in 50-mL centrifuge tubes (Corning Costar, Corning, New York). Twelve hours after fertilization, the number of trochophore larvae per mL counted in a Sedgewick-Rafter chamber. Egg samples (1 mL) from the treatments above were used to test the effect of cryoprotectant on the viability of eggs. A FDA stock solution was prepared that contained 1 mg of dye (6×10^{-3}M) per mL

of acetone, and was stored in the freezer (-20°C). To determine the viability of eggs, 1-mL egg samples were placed in 1.5-mL microcentrifuge tubes (United Scientific Products, San Leandro, California) and 1-μL of stock solution was added per mL of sample yielding a final concentration of fluorescein diacetate of 6×10^{-6} M. Stained samples were incubated for 15 min and eggs were examined using a fluorescent microscope (Microphot-SA, Nikon Inc. Garden City, New York) equipped with a high-pressure mercury lamp (Model HB 10110AF) and an excitation filter (bypass 450-490 nm) for green fluorescence. A sample of 200 eggs was examined per treatment and the number of fluorescent and non-fluorescent eggs was recorded.

Cryopreservation of eastern oyster eggs

Approximately 3×10^6 eggs per female were obtained from two ripe females (as described in Appendix A) and ~4,000 eggs per female were suspended in ASW, 0.5 M DMSO, 2 M DMSO, or 1.75 M DMSO plus 0.25 M sucrose. An average of 500 eggs was placed in 0.25-mL French straws (IMV International Corp. Minneapolis, Minnesota) and were equilibrated for 5 min. After equilibration, the straws were plunged directly into liquid nitrogen (fast cryopreservation) or frozen in a controlled-rate freezer (Kryo 10 series II, Planer products, Sunbury-on-Thames, United Kingdom) at a rate of -1.5°C per min until reaching a final temperature of -30°C which was held for 5 min before plunging into liquid nitrogen (slow freezing) (Figure A.2). After 2 days, the straws were thawed in a waterbath at 35°C for 10 sec. Eggs were collected on a 10-μ Nitex screen and were washed with filtered ASW for 10 min. A sample of ~200 eggs per treatment were placed in microcentrifuge tubes and stained with 6μM FDA for 10 min. Eggs were examined by fluorescent microscopy and the number of fluorescent and non-fluorescent eggs was recorded.

RESULTS

Flow cytometric techniques

Standard curves were generated for sperm viability and mitochondrial function. The results obtained for Sybr-14 and PI indicated that 91% of total variability was explained by the regression (Figure 6.2) (Table 6.4).

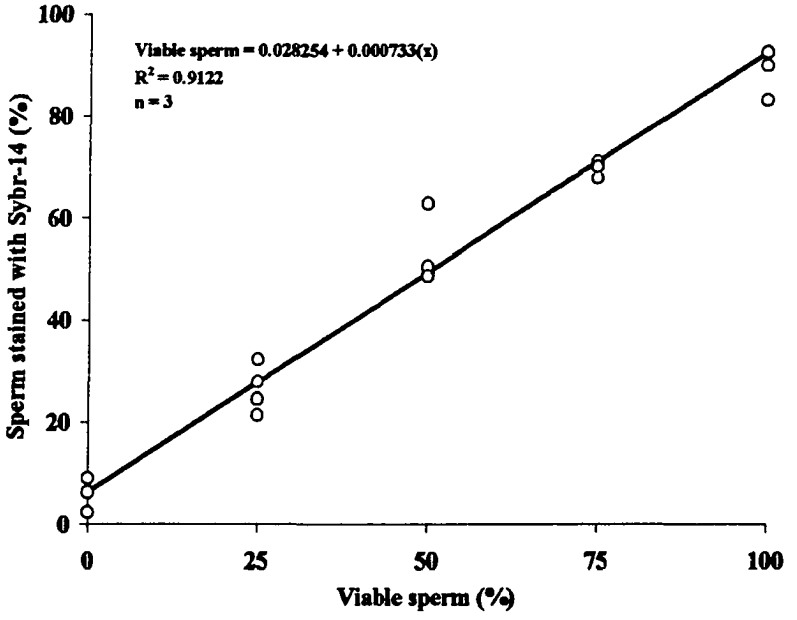

Figure 6.2 Linear regression of eastern oyster sperm stained with Sybr-14 and PI for different percentages of viable sperm.

Table 6.4 Statistical analysis of the linear regression of eastern oyster sperm stained with Sybr-14 and propidium iodide. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	P
Model	1	0.010	135	--	0.0001
Error	13	0.001	--	--	--
Corrected total	14	--	--	--	--
Intercept	1	--	--	7.313	0.0001
Sybr-14 and PI	1	--	--	11.623	0.0001

The results line obtained for R123 and PI indicated that 83% of total variability was explained by the regression (Figure 6.3) (Table 6.5) (Table D.1).

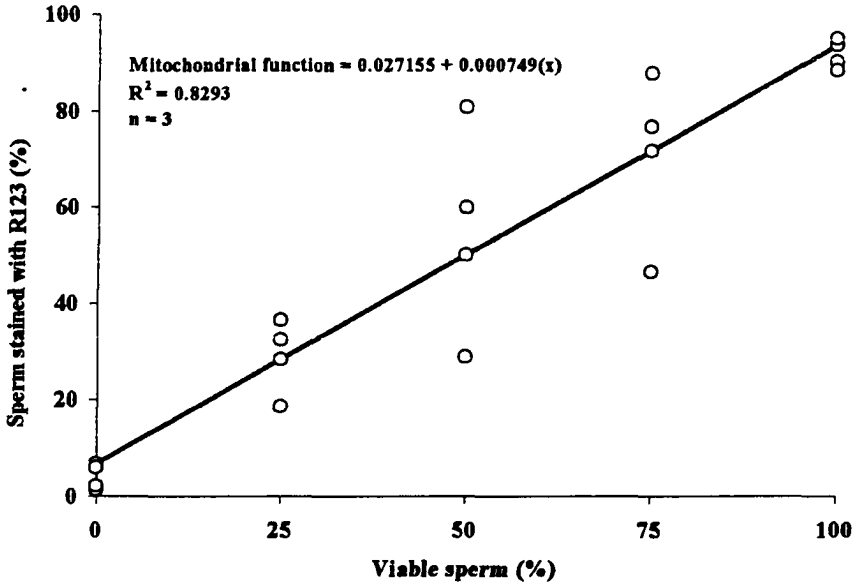

Figure 6.3 Linear regression of eastern oyster sperm stained with R123 and PI for different percentages of viable sperm.

Table 6.5 Statistical analysis for the linear regression of eastern oyster sperm stained with rhodamine 123 and propidium iodide. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	P
Model	1	0.011	63.171	--	0.0001
Error	13	0.002	--	--	--
Corrected total	14	--	--	--	--
Intercept	1	--	--	4.708	0.0004
R123 and PI	1	--	--	7.948	0.0001

When viewed by fluorescent microscopy, motile sperm displayed a bright green fluorescence after staining with Sybr-14. Also, mitochondria of motile sperm stained with R123 displayed a bright green fluorescence. Non-viable sperm (heat-treated) stained with PI showed a bright red fluorescence. Mixtures of viable and non-viable sperm displayed

dual fluorescence (green and red). Therefore microscopic observations helped to confirm the results obtained from the flow cytometer (Fig 6.4).

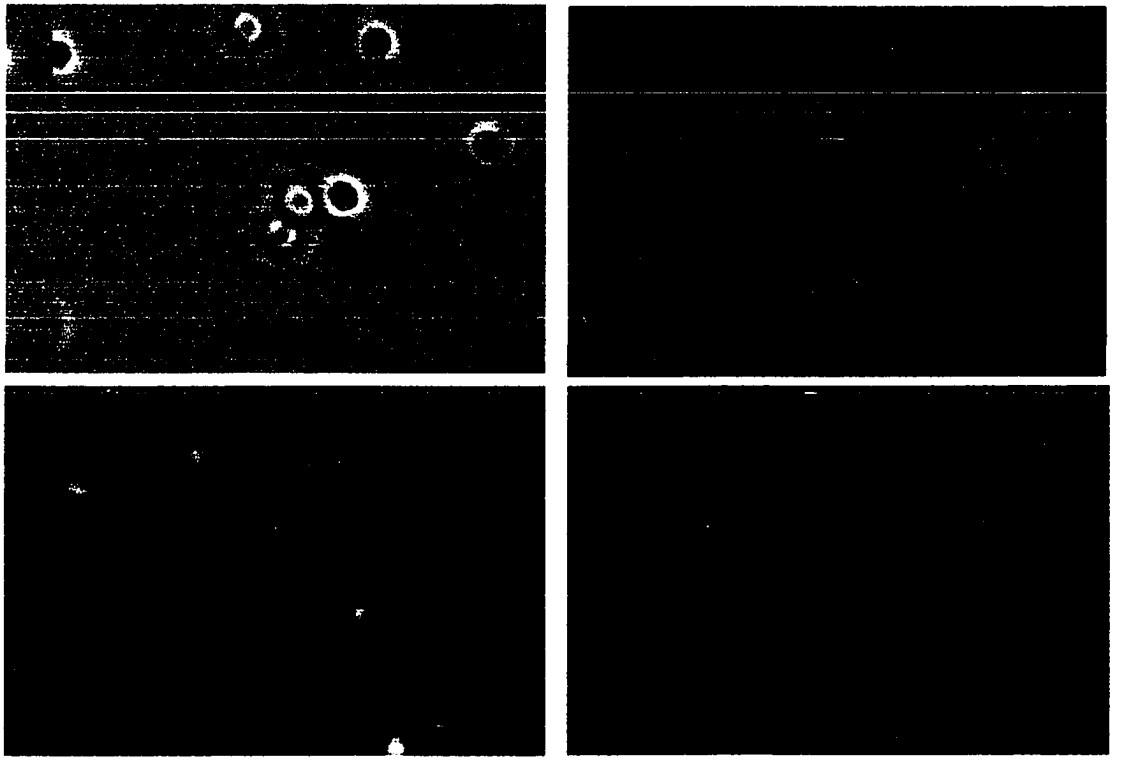

Figure 6.4 Eastern oyster sperm stained with different fluorescent dyes. Upper left panel, unstained sperm (phase-contrast microscopy); upper right, viable sperm stained with rhodamine 123; bottom left, viable sperm (green) stained with Sybr-14 and non-viable sperm (red) stained with propidium iodide; and bottom right, non-viable sperm stained with propidium iodide.

Flow cytometric analysis of thawed sperm

The highest percentages of sperm staining green with Sybr-14 were for cryopreservation with 10% propylene glycol and thawing at 25°C (38% viable sperm) and for cryopreservation with 10% propylene glycol and thawing at 70°C (30% viable sperm). The highest percentages of sperm stained with R123 were for cryopreservation with 5% propylene glycol and thawing at 25°C (68% viable) and for cryopreservation with 10% propylene glycol and thawing at 70°C (48%) (Fig 6.5).

A significant correlation was found among motility, staining with Sybr-14 and PI, cryoprotectant concentration and thawing temperature ($\underline{P} = 0.0004$). A partitioned sum of squares analysis revealed that 57% of the correlation was attributable to motility and the effectiveness of Sybr-14 and PI to stain viable and non-viable sperm ($\underline{P} = 0.0016$) (Table 6.6). A significant correlation was found among motility, staining with R123 and PI, and thawing temperatures ($\underline{P} = 0.0006$). A partitioned sum of squares analysis revealed that 60% of the correlation was attributable to motility and the effectiveness of R123 and PI to stain viable and non-viable sperm ($\underline{P} = 0.0027$) (Table 6.7).

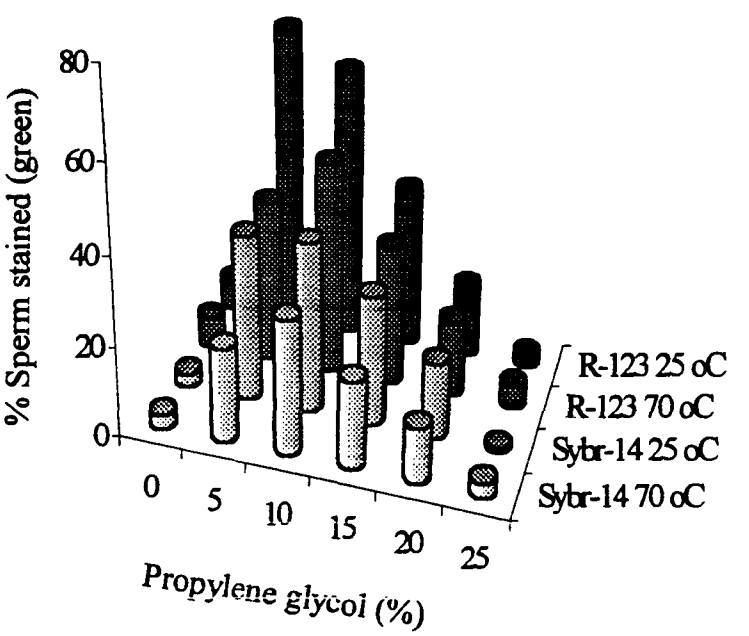

Figure 6.5 Percentages of viable eastern oyster sperm (green) stained with Sbry-14 or R123 after cryopreservation with propylene glycol (0%, 5%, 10%, 15%, 20% or 25%) and thawing at 25°C or 70°C.

A weak correlation was found among fertilizing ability, staining with Sybr-14 and PI, and thawing temperature ($\underline{P}$ = 0.0550). However, a partitioned sum of squares analysis revealed that 47% of the correlation was attributable to fertilizing ability and the effectiveness of Sybr-14 and PI to stain viable and non-viable sperm ($\underline{P}$ = 0.0264) (Table 6.8). A weak correlation was found among fertilizing ability, staining with R123 and PI, and thawing temperature ($\underline{P}$ = 0.0363). A partitioned sum of squares analysis revealed that 61% of the correlation was attributable to fertilizing ability and the effectiveness of R123 and PI to stain viable and non-viable sperm ($\underline{P}$ = 0.0152) (Table 6.9).

Table 6.6 Sperm were analyzed using a multiple regression analysis to relate dye effectiveness (Sybr-14 and PI), cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%), male (n = 3) or thawing rate (25°C or 70°C) with motility. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	$\underline{P}$
Model	4	0.363	6.972	--	0.0004
Error	31	0.404	--	--	--
Corrected total	35	--	--	--	--
Intercept	1	1.006	--	-0.413	0.6822
Sybr-14 and PI	1	0.207	--	3.455	0.0016
Cryoprotectant	1	0.064	--	-2.209	0.0347
Thawing rate	1	0.066	--	2.219	0.0339
Male	1	0.027	--	1.427	0.1636

Table 6.7 Sperm were analyzed using a multiple regression analysis to relate dye effectiveness (R123 and PI), cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%), male (n = 3) or thawing rate (25°C or 70°C) with motility. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	P
Model	4	0.350	6.520	--	0.0006
Error	31	0.416	--	--	--
Corrected total	35	--	--	--	--
Intercept	1	1.006	--	-0.676	0.5040
R123 and PI	1	0.211	--	3.259	0.0027
Cryoprotectant	1	0.046	--	-1.854	0.0733
Thawing rate	1	0.058	--	2.060	0.0479
Male	1	0.035	--	1.621	0.1151

Table 6.8 Sperm were analyzed using a multiple regression analysis to relate dye effectiveness (Sybr-14/ PI), cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%), male (n = 3) or thawing rate (25°C or 70°C) with fertilizing ability. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	P
Model	4	0.874	2.603	--	0.0550
Error	31	2.603	--	--	--
Corrected total	35	--	--	--	--
Intercept	1	1.209	--	0.836	0.4096
Sybr-14 and PI	1	0.414	--	2.332	0.0264
Cryoprotectant	1	0.105	--	-1.037	0.3077
Thawing rate	1	0.107	--	1.163	0.2538
Male	1	0.248	--	-1.719	0.0955

Table 6.9 Sperm were analyzed using a multiple regression analysis to relate dye effectiveness (R123 and PI), cryoprotectant concentration (0%, 5%, 10%, 15%, 20% and 25%), male (n = 3) or thawing rate (25°C or 70°C) with fertilizing ability. Data were arcsine square-root transformed before analysis.

Source of variation	df	SS	F	T	P
Model	4	0.955	2.933	--	0.0363
Error	31	2.523	--	--	--
Corrected total	35	--	--	--	--
Intercept	1	1.209	--	0.450	0.6556
R123 and PI	1	0.578	--	2.569	0.0152
Cryoprotectant	1	0.056	--	-0.755	0.4557
Thawing rate	1	0.099	--	1.119	0.2719
Male	1	0.221	--	-1.647	0.1096

Toxic effect of cryoprotectant solutions and cryopreservation of eastern oyster eggs

The highest osmotic pressures were found in the solutions of DMSO plus sucrose (Table 6.10). Fluorescein diacetate (FDA) appeared to be a good indicator of eggs quality. Stained eggs were fertilized with fresh sperm and developed normally. This indicated that FDA did not have toxic effects on the survival or development of embryos (Fig 6.6). DMSO (0.88 M and 1.75 M) and sucrose (0.12 M and 0.25 M) were the least toxic to eggs. The fertilization rates of these eggs were equal to these of control treatments, and the larvae produced developed normally. Eggs suspended in propylene glycol yielded the lowest percent fertilization (1- 2%). This indicated that propylene glycol was not a suitable cryoprotectant for eastern oyster eggs. The experiment was terminated when larvae were at D-stage (Table 6.11).

Table 6.10 Osmotic pressure of solutions used for suspension of eastern oyster eggs. The highest value that the vapor pressure osmometer could detect was 2,000 mOsmol/kg.

Cryoprotectant (Molarity)	mOsmol/kg (mean ± SD)
Control (no cryoprotectant)	519 ± 0
Dimethyl sulfoxide (0.86 M) and sucrose (0.14 M)	1,934 ± 92
Dimethyl sulfoxide (1.75 M) and sucrose (0.25 M)	> 2,000
Dimethyl sulfoxide (2.63 M) and sucrose (0.38)M	> 2,000
Dimethyl sulfoxide (3.50 M) and sucrose (0.50 M)	> 2,000
Propylene glycol (0.86 M) and sucrose (0.14 M)	1,499 ± 52
Propylene glycol (1.75 M) and sucrose (0.25 M)	1,874 ± 29
Propylene glycol (2.63 M) and sucrose (0.38 M)	1,857 ± 12
Propylene glycol (3.50 M) and sucrose (0.50 M)	> 2,000

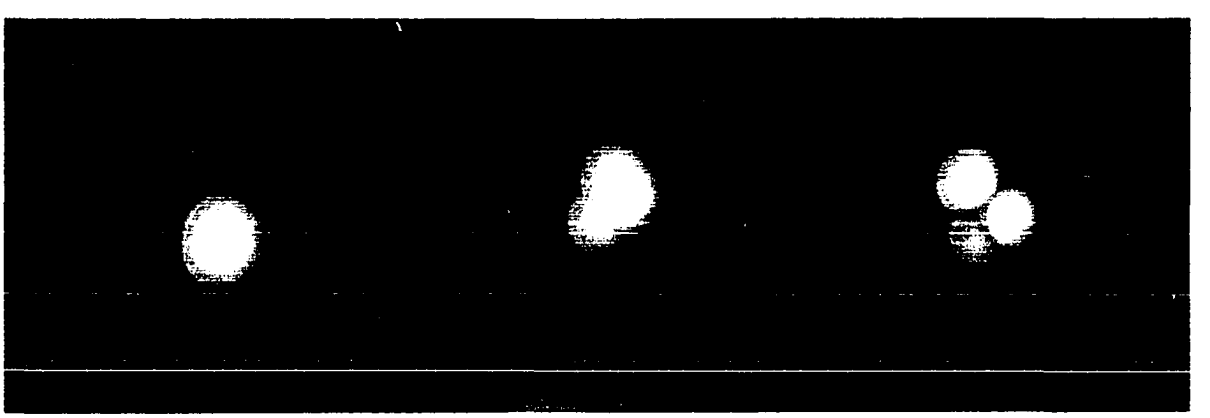

Figure 6.6 Left panel, fertilized egg (first polar body); middle, two-cell stage embryo; right, four-cell stage embryo.

Table 6.11 Eastern oyster eggs from different treatments stained with fluorescein diacetate (FDA) and the total larvae produced after fertilization. Positive, eggs stained with FDA; negative, unstained eggs; DMSO, dimethyl sulfoxide; PG, propylene glycol; S, sucrose.

Cryoprotectant (Molarity)	FDA			Larvae produced	
	Negative (number)	Positive (number)	(%)	Total (number)	Fertilization (%)
Control (no cryoprotectant)	27	173	87	878	50
DMSO (0.86 M) and S (0.14 M)	13	187	94	880	50
DMSO (1.75 M) and S (0.25 M)	36	164	82	880	50
DMSO (2.63 M) and S (0.38)M	42	158	79	374	21
DMSO (3.50 M) and S (0.50 M)	145	55	28	303	17
PG (0.86 M) and S (0.14 M)	164	36	18	28	2
PG (1.75 M) and S (0.25 M)	137	63	32	28	2
PG (2.63 M) and S (0.38 M)	132	68	34	32	2
PG (3.50 M) and S (0.50 M)	105	95	48	19	1

Before cryopreservation, the treatment that yielded the highest percentage of fluorescent eggs was 2M DMSO (165 eggs) (Table 6.13 and D.5). The cooling rate that yielded the highest average number of fluorescent eggs (14) (Table 6.14 and D.6) was the slow cooling rate (-1.5°C per min).

Table 6.12 Osmotic pressure of solutions used to cryopreserve eastern oyster eggs

Solution (molarity)	Osmotic pressure (mOsmol/kg)
Dimethyl sulfoxide (0.5 M)	1,103
Dimethyl sulfoxide (2 M)	1,927
Dimethyl sulfoxide (1.75 M) and sucrose (0.25 M)	> 2,000

Table 6. 13 Staining of eastern oyster eggs with fluorescein diacetate (FDA) after suspension in three different cryoprotectant solutions before cryopreservation. Positive, eggs stained with FDA; negative, unstained eggs; DMSO, dimethyl sulfoxide.

Treatment	FDA		Number fertilized	Percent fertilized
	Positive (number)	Negative (number)		
Control (no cryoprotectant)	140	60	550	31
DMSO (0.5 M)	137	63	0	0
DMSO (2 M)	165	35	0	0
DMSO (1.75 M) and sucrose (0.25M)	145	55	0	0

Table 6.14 Staining of eastern oyster eggs with fluorescein diacetate (FDA) after thawing. Fast, eggs plunged directly in liquid nitrogen; slow, eggs cooled at –1.5°C per min; positive, eggs stained with fluorescein diacetate; negative, unstained eggs; DMSO, dimethyl sulfoxide.

Treatment (molarity)	Cooling rate			
	Fast		Slow	
	Positive (number)	Negative (number)	Positive (number)	Negative (number)
DMSO (0.5 M)	0	200	14	186
DMSO (2 M)	0	200	0	200
DMSO (1.75 M) and sucrose (0.25 M)	0	200	0	200

DISCUSSION

It has been reported that non-motile sperm can fertilize oocytes of mammals if injected (Kuretake et al. 1996). In our study, motility and fertilizing ability of sperm were equally correlated with the effectiveness of R123 to detect functional mitochondria. Motility was directly correlated with the effectiveness of Sybr-14 to detect viable sperm, but fertilizing ability of sperm had a weak correlation. Oyster sperm have an acrosome, which is a lysosome-like vesicle located at the tip of the sperm head that plays an

important role in the fertilization. One of the possible reasons for that weak correlation is that the acrosomes may have undergone a spontaneous reaction or received structural damage which preventing entry of the sperm into the egg. Thus, it is possible to find motile sperm with damaged acrosomes that that are not capable of fertilizing eggs. The dyes used in this study responded to plasma membrane damage and functionality of mitochondria. Dyes to evaluate acrosome reaction were not used in this work. Although techniques to evaluate acrosome reaction have been developed for mammals. Normarski optics and differential interference contrast (DIC) microscopy have been useful for evaluation of acrosomal morphology in bovine sperm (Steinholt et al. 1991), and flow cytometry has been used to evaluate acrosome reaction using a fluorescent-labeled, lectin Pisum sativum agglutinin (PSA) (Graham et al. 1990). Techniques to evaluate the acrosome reaction as a parameter to determine fertilizing ability of oyster sperm have not been reported. The use of DIC microscopy combined with image analysis may be of utility, and the possible use of PSA for flow cytometric analysis should be investigated for oyster sperm.

The most difficult problem in the cryopreservation of gametes of aquatic organisms has been the cryopreservation of unfertilized eggs. Although efforts to cryopreserve oyster eggs have been attempted since at least 1988, no success has been reported (Chen et al. 1989; Naidenko 1997). Indeed, despite 40 years of effort no eggs of aquatic species have been successfully cryopreserved. The most common problem in the cryopreservation of mammalian oocytes has been osmotic injury (Isachenko et al 1998). In this study, the osmotic pressure of solutions containing propylene glycol and sucrose were lower than those of solutions of DMSO and sucrose; however, better fertilization was found in eggs suspended in DMSO and sucrose.

There are other type of injuries such as loss of cortical granules, disruption of cytoskeleton or chromosomal damage that are not easily observed and require sophisticated techniques to be identified. These damages may be related to the type and concentration of cryoprotectant and to cooling rates. The effect of DMSO on cellular structure and cytoskeletal organization has been examined in detail. In mammalian oocytes, DMSO is responsible for shifting the polymerizing center for microtubule organization from the chromosomes to the material around the centrioles. Propylene glycol has been shown to induce microtubule proliferation and disorganization of the meiotic spindle in oocytes of mouse (Maro et al. 1992) and rabbit (Vincent et al. 1989). Propylene glycol was chosen for our experiment due to its successful cryopreservation of human oocytes with subsequent normal pre-implantation development and the establishment of viable pregnancies (Van Blerkom 1991). However, fertilization and development of eastern oyster eggs suspended in propylene glycol before cryopreservation were inferior to those of eggs suspended in DMSO. Propylene glycol has been shown to induce parthenogenetic activation of mouse oocytes (Shaw and Trounson 1989), and exposure of rabbit oocytes to propylene glycol at 1.5 M to 2 M resulted in complete loss of microfilaments. After fertilization, the effect of propylene glycol on zygote actin microfilaments depended on the species (Vincent et al. 1990). However, in mouse oocytes, exposure to propylene glycol at doses as high as 1 M did not disrupt the general organization of microfilaments around the cortex (Maro et al. 1992). In mammalian oocytes, cooling rate and DMSO have each been shown to modify the properties of the zona pelucida and, by so doing, to influence the fertilizing ability of oocytes. Membrane hardening, which usually occurs after fertilization, is associated with cortical granule release (Vincent and Johnson 1992). Thus, significant reduction in cortical granule

number has been observed in the cortex of mouse oocytes treated with 1.5 M DMSO at room temperature (Schalkoff et al. 1989). Moreover, DMSO and propylene glycol can each induce a significant premature exocytosis of cortical granules from the human oocyte (Schalkoff et al. 1989).

Human oocytes are more sensitive to initial cooling than are those of mouse (Vincent and Johnson 1992). Therefore, cooling sensitivity may be species-specific and accordingly protocols designed for one group of organisms will not necessarily work for other groups. The elements of the cytoskeletal system have been shown to be sensitive to chilling (Vincent and Johnson 1992). In cell complexes, connections such as those among reticulum, globules and mitochondria may be destroyed after the oocyte is cooled or frozen (Sathananthan et al. 1992; Sathananthan 1994). The interaction between the lipid phase of cells and elements of the cytoskeleton is complex and confers another problem to the process of cryopreservation. Hardening of the lipids could cause deformation and disruption of the cytoskeleton, which plays an important role in the process of cell division. Techniques have been developed to eliminate this problem. One of these techniques is to polarize oocyte and remove the cytoplasmic lipids (Nagashima et al. 1996). However, this technique results in some physiological problems, because lipids (yolk) are the source of energy for the development of the fertilized egg (Isachenko et al. 1998). It was suggested that the chemical cytochalasin B can be used to resolve this problem. Cytochalasin B has a specific reversible effect on cytoskeletal elements making them more flexible and less susceptible to damage by cryopreservation (Isachenko et al. 1998).

In humans and mice, the stage of the oocyte can influence cryopreservation success. Immature oocytes can withstand the changes in nuclear, nucleolar and cytoplasmic organization associated with vitrification. In contrast, developmentally lethal injury may be more likely to occur during cryopreservation of mature oocytes (Van Blerkom and Davis, 1994). Embryos can be routinely produced from bovine ovarian oocytes. Such in vitro-derived embryos appear to be significantly more sensitive to low temperatures and cryopreservation than are in vivo-derived embryos (Leibo et al. 1996). In oysters, oocytes taken directly from the gonad are at the first prophase of meiosis. They undergo germinal vesicle breakdown spontaneously in natural seawater or by application of serotonin, and are arrested again at the first metaphase of meiosis (Osanai 1985). By insemination, oyster eggs are fertilized at the first prophase or the first metaphase and can even be fertilized at the second metaphase of meiosis (Osanai 1994). If the developmental stages of oyster eggs are related with the success of cryopreservation, as in human or mouse oocytes, careful collection or synchronization procedures should be considered. In this study, eggs were obtained from stripped females and it is possible that they were not in an optimum stage to be cryopreserved. In the future, eggs in different stages should be studied to examine the effects of cryopreservation. As done for mammals, studies on the effects of cryopreservation on cellular organization, chromosomes and developmental potential of oyster eggs should be performed. Many hypotheses have been produced to explain the problems related with the cryopreservation of mammalian oocytes. These hypotheses could provide suggestions for advances in cryopreservation of oyster eggs.

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CHAPTER 7
SUMMARY AND CONCLUSIONS

Culture of the eastern oyster, Crassostrea virginica, is of economic importance to the state of Louisiana. In addition to contributing to the national consumption of oysters, it is also linked to the culture and heritage of the state (Pausina 1988). The Louisiana industry depends each year on public oyster beds for supplies of seed oysters, although such natural production is cyclical. Lack of consistent seed supply for oyster farming is a significant detriment to the economic development of the oyster industry (Supan and Wilson 1993). Practical applications arising from the ability to store gametes and larvae in the frozen state can be foreseen. The potential of long-term storage of gametes and larvae of aquatic organisms for practical applications and for research is beginning to be appreciated. Reliable methods of storage would have immediate application in the rapid and cheap transportation of frozen material. The distance over which fresh gametes and larvae could be transported is currently limited by the time for which they can be stored. Cryopreservation would abolish such limitations. A period of storage before transfer would provide time for testing of parents for diseases and production characteristics. The maintenance of disease-free organisms as sources of gametes and embryos for export would become feasible, and storage of gametes and embryos could prove to be valuable in the conservation of rare breeds and of organisms of potential genetic value (Fig. 7.1). Applications such as these, however, are dependent on obtaining high survival rates after thawing.

Cryopreservation of gametes and larvae has begun to be applied to aquaculture based on the benefits that can be obtained from this technique. In mollusks, most of the work has focussed on the Pacific oyster, C. gigas. Although the eastern oyster is considered to

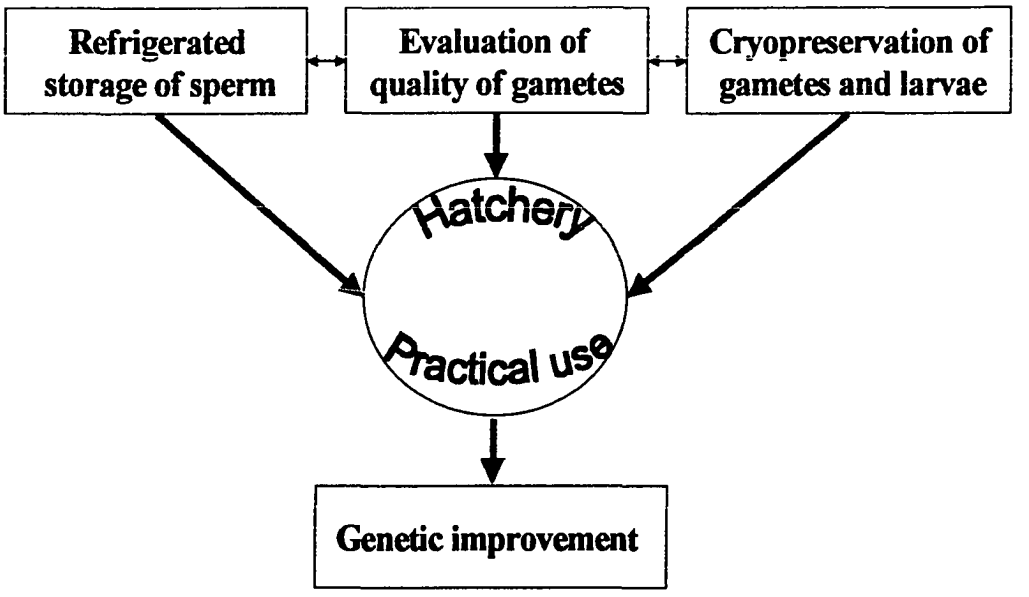

Figure 7.1 Genetic improvement will result after the elements of the cryopreservation process are integrated and applied.

be an important species in the United States, little attention has been directed to the cryopreservation of gametes or larvae of this species.

The goals of this work were to not only develop techniques for short-term storage of sperm and cryopreservation of gametes and larvae, but also to evaluate potential application in aquaculture to assist with hatchery production. It was found that sperm stored in calcium-free Hanks' balanced salt solution (C-F HBSS) yielded the highest larval survival when it was used to fertilize fresh eggs. In refrigerated storage, undiluted sperm retained motility longer. Bacterial contamination was observed and could be a reason for reduced motility during refrigerated storage due to oxygen depletion.

Dimethyl sulfoxide is the most used cryoprotectant for oyster sperm. However, it was found that 10% propylene glycol was more effective as a cryoprotectant for eastern oyster sperm. Addition of sucrose did not seem to affect cryoprotection, and in some cases it was associated with decreased sperm motility. Propylene glycol has been used

for the cryopreservation of larvae of the Pacific oyster (Gwo 1995). Eastern oyster larvae also responded well to propylene glycol. The feasibility of using 5-mL macrotubes instead of the conventional 0.5-mL straws was investigated and it was found that for practical use, the largest volume was used for hatchery or commercial production.

One of the most exciting results of this work was the first-time production of spat from thawed sperm and thawed larvae, demonstrating the feasibility of cryopreservation for commercial application.

If little attention has been placed on the cryopreservation of gametes and larvae of the eastern oyster, even less attention has been placed on evaluation of gamete quality. Methodological alternatives were sought to the usual observations of motility and fertilizing ability of thawed sperm. Flow cytometry was used to evaluate the quality of thawed sperm with combinations of the fluorescent dyes Sybr-14 and propidium iodide (PI), and rhodamine 123 (R123) and PI. The effectiveness of flow cytometric techniques was evaluated by correlating motility and fertilizing ability of thawed sperm with the dyes used to evaluate membrane integrity. Flow cytometry was rapid and effective. However, more attention is required for analysis of other important structures. Eastern oyster sperm possess an acrosome necessary for fertilization. Evaluation of acrosomal integrity using flow cytometric techniques would be useful because the acrosomes of oyster sperm are small and difficult to evaluate by microscopy.

Cryopreservation of unfertilized eggs has not been accomplished in aquatic organisms (Hagedorn 1997). Preliminary studies were performed to evaluate cryopreservation methods for oyster eggs. Dimethyl sulfoxide (0.88 and 1.75 M) was found to not affect fertilizing ability of eggs and could be used as cryoprotectant;

however, cooling had a considerable effect on unfertilized eggs. Thawed eggs could not be fertilized and did not yield larvae. Careful attention to temperature control throughout egg collection, examination, and handling could help to overcome the problems associated with cooling. The failure to cryopreserve eggs could be also related to damage of the cytoskeleton before freezing and after thawing.

Practical applications of cryopreservation techniques have focussed on the breeding of farm animals. During the last 40 years there has been a pronounced increase in the number of dairy and beef producers that artificially inseminate cattle. In aquatic organisms, cryopreservation of fish sperm is a reality, although cryopreservation in other animals such as birds, reptiles, amphibians and most invertebrates is lacking and many of these organisms are considered to be endangered. The use of cryopreservation techniques could be of benefit in slowing the decline in diversity of aquatic species worldwide including taxa such as the highly endangered freshwater mussels (of the family Unionidae). This trend is especially severe in developing countries. Accordingly I have included simplified and economical methods for short-term storage and cryopreservation in Appendix E.

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APPENDIX A
STANDARD OPERATIONAL PROCEDURES (SOPS)

Oyster collection

Oysters were collected from a hatchery on Grand Isle, Louisiana (29°15'12"N, 90°03'26"W) maintained by the Louisiana Sea Grant College Program, and were transported to the Louisiana State University Agricultural Center, Aquaculture Research station, Baton Rouge. Oysters were opened and inspected visually for the presence of gonad development and prominent genital canals (Supan 1996). A gonad sample was collected with a capillary tube and smeared on a glass microscope slide for examination at 200 X. Sex was identified based on the presence of eggs or sperm.

Gamete preparation

Gamete samples were removed from each oyster by the dry stripping method (Allen and Bushek 1992). The gonad was gently disrupted and gonadal material was collected with a Pasteur pipette. A 10-μL sample was removed from the gonad to measure osmolality with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah). Egg or sperm samples were placed in 50-mL beakers until suspension in an extender. After suspension, eggs were washed through a 70-μ nitex screen (Aquacenter, Leland, Mississippi), collected on a 15-μ screen, and suspended in artificial sea water at 601 mOsmol/kg (ASW 601). For sperm, samples were washed through 70-μ and 15-μ screens and motility was estimated as described below.

Motility estimation

A 10-μL sample was removed from sperm suspensions to estimate motility. The sample was mixed with 20 μL of ASW 601 on a glass microscope slide. The percentage of sperm exhibiting vigorous forward movement was estimated at 200 X using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, New York). Sperm vibrating in place were not considered to be motile. Only males with actively swimming sperm (> 90%) were selected for experimentation.

Table A.1 Ingredients of Hanks' balanced salt solution (HBSS), calcium-free HBSS (C-F HBSS) and DCSB4 solutions used to dilute oyster sperm.

	Concentration (g/L)		
Ingredient	HBSS	C-F HBSS	DCSB4*
NaCl	24.00	24.00	24.00
KCl	1.20	1.20	-
$CaC_{l2}.2H_2O$	0.48	-	0.31
$MgSO_4.7H_2O$	0.60	0.60	0.31
$Na_2HPO_4.7H_2O$	0.36	0.36	-
KH_2PO_4	0.18	0.18	-
$NaHCO_3$	1.05	1.05	-
$C_6H_{12}O_6$ (glucose)	3.00	3.00	-
Tris-HCl	-	-	2.98

*Bougrier and Rabenomanana. Aquaculture (1986, 58:277-280).

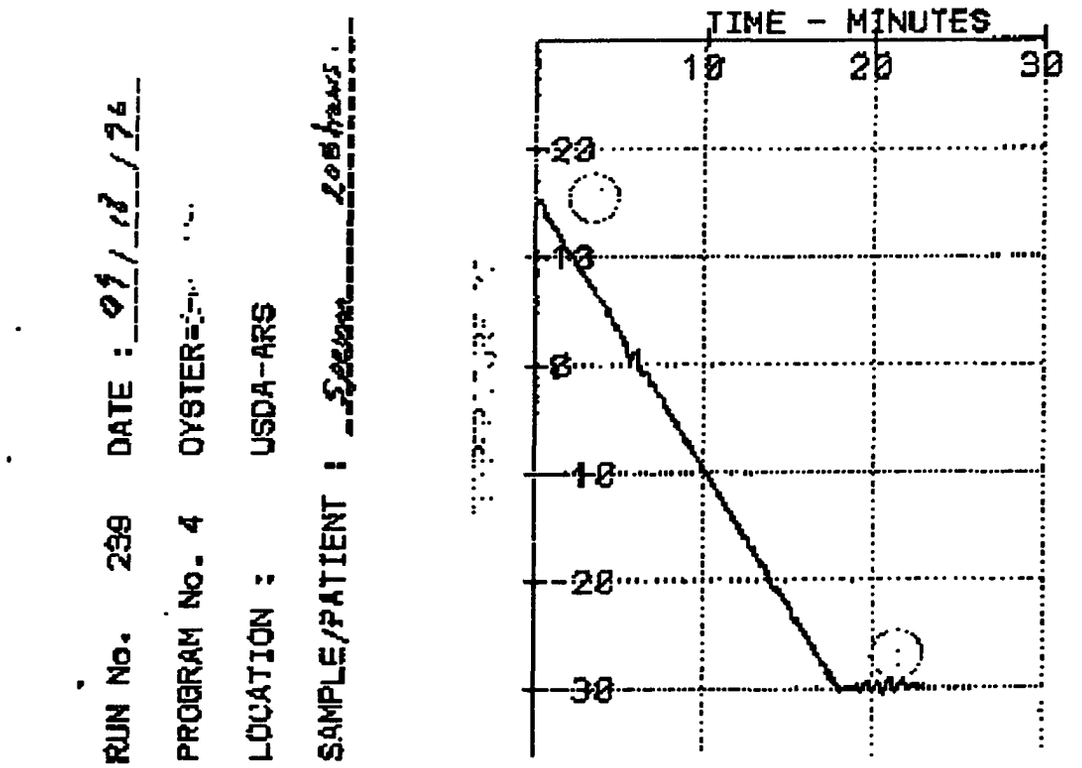

Figure A.1 Sperm and larvae of the eastern oyster were frozen in a controlled-rate freezer (Kryo 10 series II, Planer products, Sunbury-on-Thames, United Kingdom). The initial temperature was 15°C, the samples were cooled at a rate of –2.5°C per min until reaching a final temperature of -30°C which was held for 5 min before the samples were plunged into liquid nitrogen for storage.

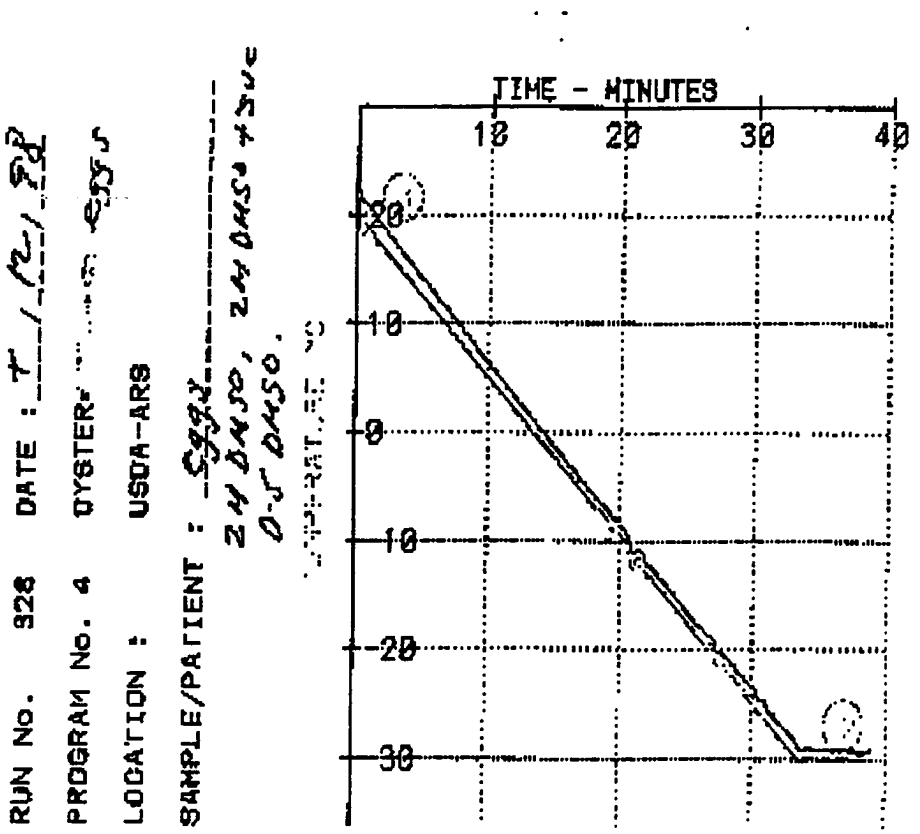

Figure A.2 Eggs of the eastern oyster were frozen in a controlled-rate freezer (Kryo 10 series II, Planer products, Sunbury-on-Thames, United Kingdom). The initial temperature was 20°C, the samples were cooled at a rate of –1.5°C per min until reaching a final temperature of -30°C which was held for 5 min and then were plunged into liquid nitrogen for storage.

APPENDIX B
UNANALYZED DATA FOR OYSTER SPERM IN CHAPTER 3

Table B.1 Percent motility of eastern oyster sperm diluted in three different extenders (Fig. 3.2).

		Motility (%)		
Extender	Day	Oyster #1	Oyster #2	Oyster #3
DCSB4	0	73	73	73
HBSS	0	80	77	77
ASW	0	80	88	87
DCSB4	1	67	63	28
HBSS	1	67	73	63
ASW	1	63	87	18
DCSB4	2	57	46	0
HBSS	2	60	77	17
ASW	2	23	83	0
DCSB4	3	1	1	0
HBSS	3	10	27	0
ASW	3	0	13	0
DCSB4	4	1	0	0
HBSS	4	2	20	0
ASW	4	0	15	0

Table B.2 Percent motility of eastern oyster sperm diluted in artificial seawater (ASW) or Hanks' balanced salt solution (HBSS) at six different dilutions (Figs. 3.3 and 3.4).

Diluent	Dilution	Day	Motility(%)		
			Oyster#1	Oyster#2	Oyster#3
ASW	Not diluted	0	99	90	99
	1:1	0	99	80	80
	1:3	0	80	95	70
	1:7	0	80	70	50
	1:15	0	70	70	60
	1:31	0	50	70	20
	Not diluted	1	99	90	70
	1:1	1	98	80	80
	1:3	1	80	95	70
	1:7	1	80	70	50
	1:15	1	60	70	30
	1:31	1	20	40	30
	Not diluted	2	99	90	70
	1:1	2	60	10	0
	1:3	2	60	20	0
	1:7	2	30	10	0
	1:15	2	0	40	0
	1:31	2	0	10	0
	Not diluted	3	80	70	60
	1:1	3	20	0	0
	1:3	3	1	20	0
	1:7	3	0	0	0
	1:15	3	0	0	0
	1:31	3	0	0	0
	Not diluted	4	70	70	0
	1:1	4	0	0	0
	1:3	4	0	0	0
	1:7	4	0	0	0
	1:15	4	0	0	0
	1:31	4	0	0	0
HBSS	Not diluted	0	99	90	99
	1:1	0	70	80	80
	1:3	0	90	80	70
	1:7	0	90	90	50
	1:15	0	20	90	60
	1:31	0	1	80	20
	Not diluted	1	99	90	70
	1:1	1	70	70	80
	1:3	1	90	70	70
	1:7	1	90	10	50
	1:15	1	20	0	30
	1:31	1	1	0	30
	Not diluted	2	99	90	70
	1:1	2	0	70	0
	1:3	2	0	70	0
	1:7	2	0	10	0
	1:15	2	0	0	0
	1:31	2	0	0	0
	Not diluted	3	0	70	60
	1:1	3	0	50	0
	1:3	3	0	02	0
	1:7	3	0	10	0
	1:15	3	0	0	0
	1:31	3	0	0	0
	Not diluted	4	70	70	0
	1:1	4	0	0	0
	1:3	4	0	0	0
	1:7	4	0	0	0
	1:15	4	0	0	0
	1:31	4	0	0	0

Table B.3 Percent motility and fertilizing ability of eastern oyster sperm suspended in five different diluents* (Fig. 3.5).

Diluent	Oyster # 1		Oyster # 2		Oyster # 3	
	Motility (%)	Larval survival (%)	Motility (%)	Larval survival (%)	Motility (%)	Larval survival (%)
ASW	70	4	75	6	70	7
ASW+G	50	22	70	17	70	4
ASW 200	20	1	10	0	5	0
HBSS	90	29	90	28	80	29
C-F HBSS	99	62	99	42	90	39

* ASW, artificial sea water; ASW+G, artificial sea water plus glycine; ASW 200, artificial sea water at 200 mOsmol/kg; HBSS, Hanks' balanced salt solution, and C-F HBSS, calcium-free HBSS

APPENDIX C
UNANALYZED DATA FOR EASTERN OYSTER SPERM IN CHAPTER 4

Table C.1 Osmotic pressure (mOsmol/kg) and motility of eastern oyster sperm suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) containing propylene glycol (PG) before cryopreservation (Fig 4.1).

	Male # 1				Male # 2				Male # 3			
	Sucrose		No sucrose		Sucrose		No sucrose		Sucrose		No sucrose	
PG (%)	Osmotic pressure	Motility (%)	Osmotic pressure	Motility (%)	Osmotic pressure	Motility (%)	Osmotic pressure	Motility (%)	Osmotic pressure	Motility (%)	Osmotic pressure	Motility (%)
0	973	80	668	90	958	10	670	99	942	30	666	99
5	1396	50	1093	90	1403	10	1057	99	1412	20	1127	99
10	1699	50	1461	90	1661	10	1450	99	1622	20	1471	99
15	1825	20	1587	50	1842	0	1612	90	1858	0	1561	90
20	1842	0	1665	0	1854	0	1643	10	1865	0	1684	1
25	1904	0	1767	0	1922	0	1732	0	1941	0	1800	0

Initial Motility: Average: 96 ± 5%; Male 1, 90 %; Male 2, 99 %; Male 3, 99 %.

Table C.2 Fertilizing ability and motility of eastern oyster sperm suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) containing propylene glycol (PG) after thawing at 25°C (Fig 4.2).

	Male # 1				Male # 2				Male # 3			
	Sucrose		No sucrose		Sucrose		No sucrose		Sucrose		No sucrose	
PG (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)
0	1	0	0	0	1	> 1	0	0	5	> 1	0	0
5	1	26	1	> 1	5	21	3	0	5	2	2	3
10	1	0	2	7	10	41	20	77	5	4	5	3
15	1	1	5	1	5	1	20	9	10	0	15	0
20	0	0	0	0	0	0	1	0	1	0	2	0
25	0	0	0	0	0	0	1	0	0	0	1	0

Initial Motility: Average: 96 ± 5%; Male 1, 90 %; Male 2, 99 %; Male 3, 99 %.

Table C.3 Fertilizing ability and motility after thawing at 70°C of eastern oyster sperm suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) containing propylene glycol (PG) (Fig 4.3). The control treatment yielded 31% survival to trochophore larvae.

	Male # 1				Male # 2				Male # 3			
	Sucrose		No sucrose		Sucrose		No sucrose		Sucrose		No sucrose	
PG (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)	Motility (%)	Larvae (%)
0	1	0	0	0	1	4	0	0	5	0	0	0
5	5	17	5	0	5	> 1	3	63	5	2	2	26
10	10	100	2	100	15	49	25	68	10	2	10	57
15	10	1	15	1	25	45	25	43	25	0	25	15
20	0	0	2	0	0	0	1	0	1	0	3	0
25	0	0	0	0	0	0	0	0	0	0	0	0

APPENDIX D
UNANALYZED DATA FOR OYSTER SPERM AND EGGS IN CHAPTER 6

Table D.1 Percentages of viable and non-viable sperm of the eastern oyster stained with Sybr-14 or R123.

Ratio of viable and non-viable sperm	Sperm stained with Sybr-14			Sperm stained with R123		
	Male # 1	Male #2	Male # 3	Male # 1	Male #2	Male # 3
100:0	90	83	93	90	88	95
75:25	70	68	84	47	77	88
50:50	63	49	51	29	60	81
25:75	21	24	32	19	32	36
0:100	9	2	2	6	1	2

Table D.2 Eastern oyster sperm suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) containing propylene glycol (PG) after thawing at 25°C, staining with Sybr-14 or R123 and analysis by flow cytometry. The percentages represent values gated under green fluorescence and are considered to represent viable sperm.

	Male # 1		Male # 2		Male # 3	
PG (%)	Sybr-14 (%)	R123 (%)	Sybr-14 (%)	R123 (%)	Sybr-14 (%)	R123 (%)
0	2	7	4	7	3	7
5	29	63	35	71	48	70
10	19	49	38	61	58	72
15	19	25	36	57	32	27
20	19	19	0	0	31	29
25	2	6	0	2	0	0

Table D.3 Easter oyster sperm suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) containing propylene glycol (PG) after thawing at 70°C, staining with Sybr-14 or R123 and analysis by flow cytometry. The percentages represent values gated under green fluorescence and are considered to represent viable sperm.

	Male # 1		Male # 2		Male # 3	
PG (%)	Sybr-14 (%)	R123 (%)	Sybr-14 (%)	R123 (%)	Sybr-14 (%)	R123 (%)
0	5	8	1	6	3	6
5	0	3	20	54	42	52
10	3	38	28	58	30	47
15	22	28	18	36	19	30
20	28	37	9	11	0	2
25	9	11	0	2	0	2

Table D.4 Unanalyzed data for eggs of the eastern oyster suspended in three different cryoprotectant solutions before cryopreservation. Positive, eggs stained with fluorescein diacetate (FDA); negative, unstained eggs.

Treatment	FDA		Fertilization	
	Positive	Negative	Number larvae	(%)
Control	146	54	550	31
	133	67	725	41
DMSO (2 M)	182	18	--	--
	147	53	--	--
DMSO-SUCROSE (2 M)	136	64	--	--
	153	47	--	--
DMSO (0.5 M)	134	66	--	--
	140	60	--	--

Table D.5 Unanalyzed data for thawed eggs of the eastern oyster. Fast, eggs were plunged directly in liquid nitrogen; slow, eggs were cooled at a rate of −1.5°C per min; positive, eggs stained with fluorescein diacetate (FDA); negative, unstained eggs.

Treatment	Cooling rate			
	Fast		Slow	
	Positive	Negative	Positive	Negative
DMSO (2 M)	0	200	0	200
	0	200	0	200
DMSO-SUCROSE (2 M)	0	200	0	200
	0	200	0	200
DMSO (0.5 M)	0	200	18	182
	0	200	10	190

APPENDIX E
SIMPLIFIED AND ECONOMICAL METHODS FOR SHORT-TERM STORAGE AND CRYOPRESERVATION OF SPERM OF AQUATIC SPECIES

Finally, I describe simple and inexpensive methods that can be used in the field. Included are methods to collect sperm samples and to keep records, methods for short-term storage and cryopreservation of sperm, and sperm extender solutions that can be prepared with reagents found in drug stores, hardware stores or supermarkets. I hope this information will be useful in conservation of endangered species in developing countries.

Refrigerated storage

Sample collection. Four examples for collection of sperm can be described. 1) Whole gonads: gonads can be dissected carefully, wrapped in paper towel and placed into a resealable plastic bag (e. g., Ziploc®, S. C. Johnson & Son, Inc. Racine, Wisconsin). The gonads can be refrigerated in a portable refrigerator (Fig. E.1). This method could be used when extender is not available at the moment of collection. 2) Pieces of gonads: Gonads can be extracted and dissected into small pieces. The pieces can be placed in resealable plastic bags containing an extender. Pieces of gonads must be placed in an extender due to loss of fluids after dissection. 3) Suspension of sperm: Gonads can be extracted, macerated into a Ziploc® bag and rinsed with an extender. The sperm suspension can be collected in a clean plastic bag and can be refrigerated in the portable refrigerator. 4) Stripping: When mature males are obtained a better method to collect sperm is stripping. Samples can be collected in plastic bags and can be suspended in an extender.

Extenders. The type of extender used to suspend the sperm depends on the type of organism (saltwater or freshwater). One of the most important parameter to determine is the osmotic pressure of the solution. A useful method to determine the osmolality when an osmometer is not available is to calculate it using the formula:

$$\Pi = RTim$$

Where:

Π = Osmotic pressure of the solution

R = Universal gas constant (0.82 atm x liter/ °K x mole)

T = Absolute temperature (room temperature in °K)

i = Van t'Haff factor (identify for each ingredient)

m = solute molality (for each ingredient)

The use of this formula and details such as the Van t'Haff factor can be obtained from standard references such as Martin (1990).

Also, the type of extender is important. In developing countries, packets of electrolytic solutions could be used as extenders. Electrolytic solutions could be found in supermarkets and drugstores at low prices. In some countries such as Mexico, the packets of salts to make electrolytic solutions are distributed to the population by the government. Instructions such as preparation of the solution, and the type and quantity of salts are listed in the back of each packet. Electrolytic solutions could be used to substitute for physiological solutions such as Ringers' or Hanks' solutions. Another way to make inexpensive saline solutions would be to buy the items in drug stores or supermarkets.

Containers. The most suitable container for refrigeration of sperm could be Ziploc® plastic bags. These bags have various benefits: 1) they can be found in any supermarket; 2) they are inexpensive and come in different sizes and styles, and 3) are easy to handle and transport (Carmichael et al. 1996)

Equipment. An office refrigerator could provide an inexpensive method to store sperm where electricity is available. However, in places where there is no electricity, other kinds of equipment are necessary. Figure E.1 depicts the construction of an inexpensive portable refrigerator. The refrigerator is constructed with a styrofoam box cooler. The coolant in the portable refrigerator is dry ice which is easy to find and inexpensive in developing countries (Fig E.1). The dimensions of the box and the quantity of dry ice would depend of the quantity of samples and time of storage. The dry ice blocks should be wrapped with paper and should be place in the bottom of the box. If necessary, stands could be used to hold the samples. A thermometer should be used to determine temperature of the box. The dry ice should be replaced as needed.

Cryopreservation

Sample collection and extenders. To cryopreserve sperm, it is necessary to suspend the sperm in an extender. The procedure that should be used is similar to procedure described above for sample collection for refrigerated sperm. The same extenders used for refrigerated storage could be used to cryopreserve sperm.

Cryoprotectants. Some times sperm can be cryopreserved with methanol (e. g. Tiersch et al. 1994) Industrial-grade methanol can be found in hardware stores at low prices in developing countries. However, if the samples require another type of cryoprotectant such as dimethyl sulfoxide (DMSO), the purchase of medical-grade reagent is

unavoidable because there is no a substitute. Ethylene glycol (reagent grade) could be replaced with automobile antifreeze (coolant) because the principal component of these preparations is ethylene glycol.

Non-permeating cryoprotectants such as glucose and proteins can be found in the supermarket in the form of granulated sugar, eggs and milk.

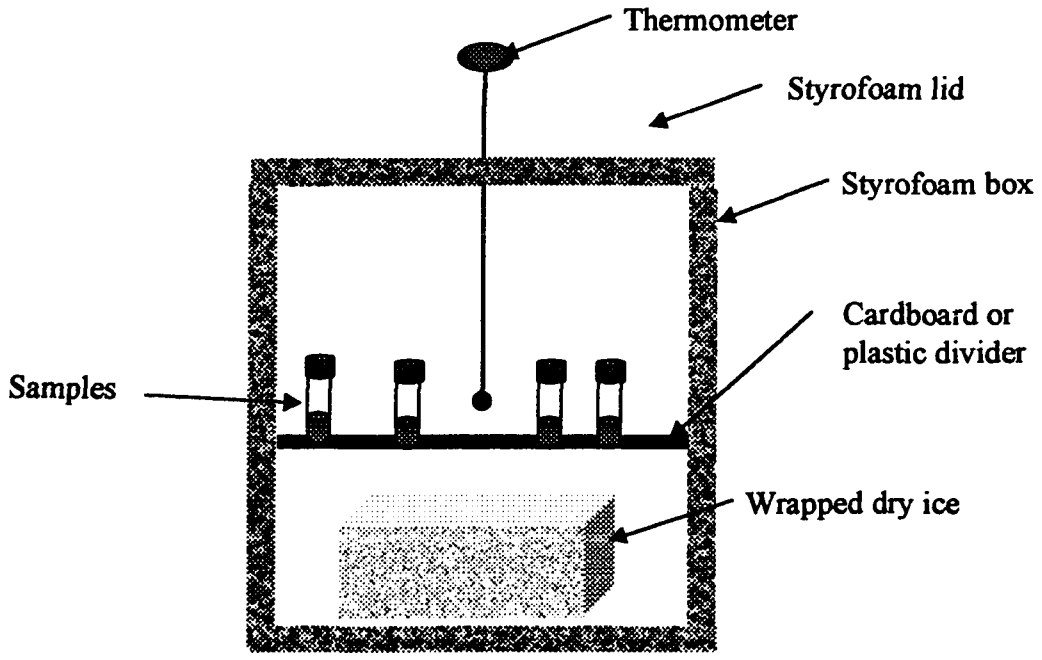

Figure E.1 Schematic diagram of a portable refrigerator for short-term storage of sperm.

Containers. Inexpensive containers for cryopreservation of sperm include small Ziploc® plastic bags and food-grade straws. When a large volume of sperm is required (10 to 20 mL of extended sperm), it could be cryopreserved in small Ziploc® plastic bags. For homogeneous cryopreservation, plastic bags containing sperm samples should be cooled on a screen (Fig E.2). For small quantities of sperm, food-grade straws are a better option. It is possible to find different sizes of drinking straws in the supermarket (from

~2.5 mL to 5 or 10 mL). Plastic syringes can be used to fill the straws and modeling clay or melting can be used to close the ends of the straws.

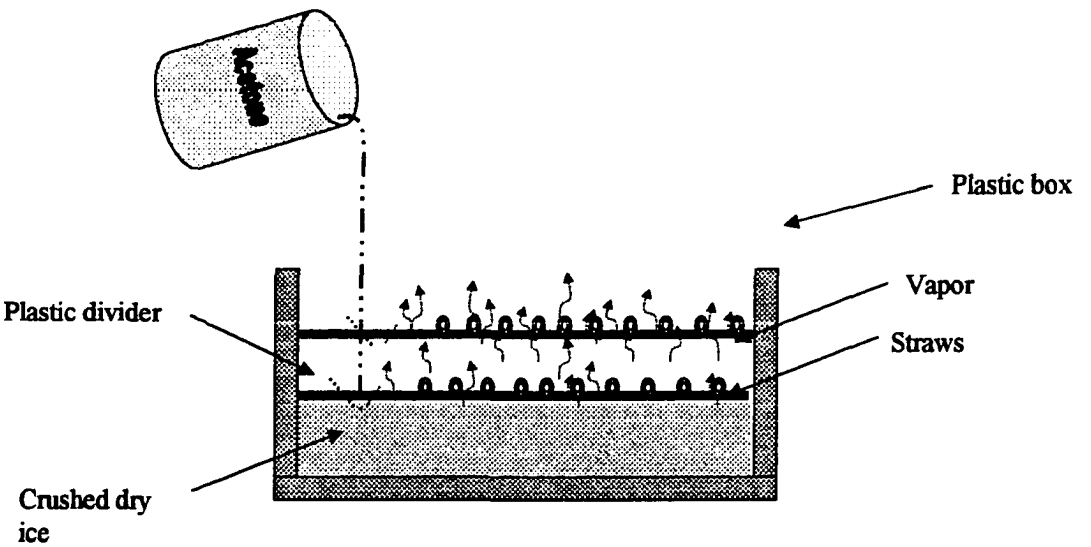

Figure E.2 Modification of a portable refrigerator to freeze sperm samples. The samples could be frozen at different heights above the dry ice to vary cooling rate.

Equipment. The portable refrigerator can also be used as a freezing chamber (Fig. E.2). Pieces of crushed dry ice should be placed in the bottom of the box. A plastic screen could be used to hold the samples. Acetone could be used when a rapid cooling rate is desired for cryopreservation. Acetone can be found at low prices in places such as drug stores, supermarkets and hardware stores. After freezing, the samples must be stored in liquid nitrogen in dewars. This part of the cryopreservation process is the most expensive, and cannot be replaced. Another method to freeze samples is to make small holes in a block of dry ice (Fig E.3). Drops of extended sperm can be placed in the holes to form small frozen pellets. As soon the pellets are frozen, they can be stored in liquid nitrogen.

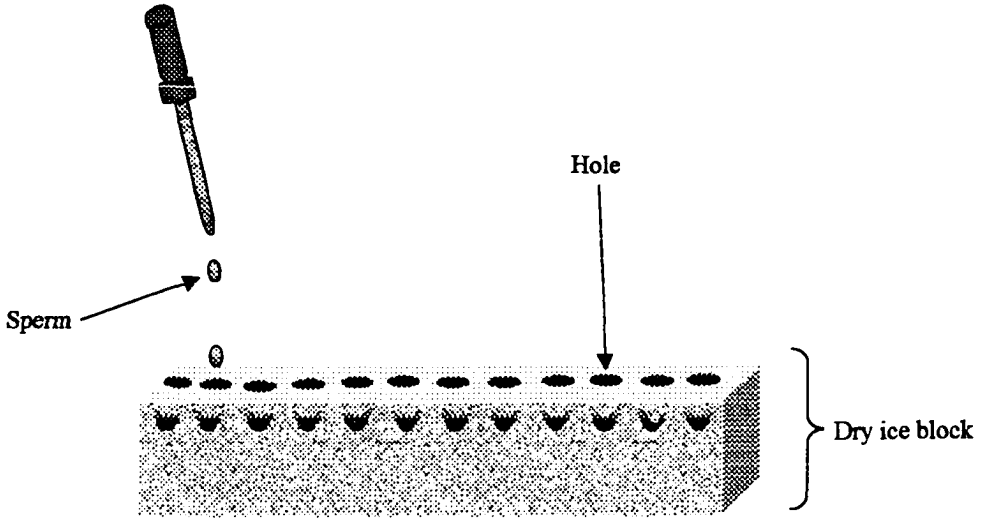

Figure E.3 An alternative method to freeze sperm as pellets.

Thawing. A simple method to thaw samples is the use of a kitchen stove. A cooking pot can be used to hot water to a desired temperature such as 40°C. A thermometer should be used to help control the thawing temperature based on the species and treatment that was used to cryopreserve the sperm.

Record keeping

Keeping good records is an important part of research. A Table containing dates, gonad condition, cryoprotectant and treatment of the sperm should be recorded. An example of a data sheet is shown in Table E.1.

Table E.1. An example data sheet for short-term storage and cryopreservation of sperm.

Date	Code*	Species	Gonad condition	Motility (%)		Cryoprotectant	Chamber temperature (°C)	Thawed sperm motility (%)	Fertilizing ability (%)**	Comments
				Initial	equilibrium					

*Code could be a fish number or collection site.

**Record fertilization value for non-frozen sperm (control) with each batch of eggs.

APPENDIX F
LETTERS OF PERMISSION

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January 14, 1999

Dr. Sandra E. Shumway
Journal of Shellfish Research
Natural Science Division
Southampton College, LIU
Southampton, New York 11968

Dear Dr. Shumway,

I am preparing my dissertation and I would like to request your permission to reproduce material from my manuscript entitled "Effect of extender solutions and dilution on motility and fertilizing ability of eastern oyster sperm" published in the Journal of Shellfish Research Vol. 17 No. 1.

With thanks,

Carmen Paniagua

permission granted with pleasure. S. Shumway

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January 14, 1999

Dr. Peter L. Steponkus
Cryo-Letters
Department of Soil, Crop
And Atmospheric Sciences
Bradfield and Emerson Halls
Ithaca, NY 14853

Dear Dr. Steponkus,

I am preparing my dissertation and I would like to request your permission to reproduce material from my manuscript number NA-117 entitled "Settlement and growth of eastern oysters produced from cryopreserved larvae" published in Cryo-Letters Vol. 19.

With thanks,

Carmen Paniagua

Permission is granted

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October 29, 1999

Dr. Terrence R. Tiersch
Aquaculture Research Station
Louisiana State University
2410 Ben Hur Rd.
Baton Rouge LA 70820

Dear Dr. Tiersch,

I am preparing my dissertation and I would like to request your permission to reproduce material from my manuscript entitled "Hatchery production of eastern oyster from cryopreserved larvae and cryopreserved sperm" that will be published in Advances in World Aquaculture, World Aquaculture Society Vol. 7.

With thanks,

Carmen Paniagua

11/1/99

Permission approved.

Terrence Tiersch

VITA

Carmen Guadalupe Paniagua-Chavez was born on July 14, 1962, in Ciudad Juarez Chihuahua, Mexico, the daughter of Carmen Chavez and Jose Paniagua. She attended The Escuela Preparatoria Federal por Cooperacion Benito Juarez in Ensenada, Baja California, Mexico, and graduated in June, 1981.

In January, 1979, she was hired as secretary by Asociacion Bajacaliforniana de Comerciantes en Automoviles, where she worked for 3 years. In June, 1981, she was hired by the High School Benito Juarez were she served as head of document control for a year.

She was admitted into the Universidad Autonoma de Baja California in September, 1981. In May, 1982, she was hired by the Centro de Investigacion Cientifica y Educacion Superior de Ensenada as a Research Assistant to work with the Pacific oyster Crassostrea gigas. In June, 1986, she graduated with a Bachelor of Science degree in Biology. The title of her thesis was "Desarrollo gonadal de la Especie Crassostrea gigas en Bahia San Quintin Baja California Mexico. ("Gonadal development of the species Crassostrea gigas in San Quintin Bay, Baja California, Mexico").

A lover of the fine arts, she enrolled in ballet and jazz dance classes. She practiced dance for 4 years and in the fall of 1987 was hired as substitute teacher to teach classical ballet for a semester in the City Cultural House in Ensenada Baja California, Mexico..

In January, 1987, she was hired as Assistant Professor by the School of Science of the Universidad Autonoma de Baja California and in January, 1988, she was hired as a science teacher in the secondary school Escuela Secundaria Diurna No. 2 where she served for 5 years.

She was admitted into Centro de Investigacion Cientifica y Educacion Superior de Ensenada in August, 1990. In January, 1993, she graduated with a Master of Science degree in oceanography with a specialization in marine ecology. The title of her thesis was "Viabilidad, Composicion Proximal y Valor Alimenticio de Dunalliella sp. Preservada por Congelamiento. ("Viability, proximate composition and nutritional value of Dunalliella sp preserved by freezing").

Upon graduation she married Manuel Segovia. They moved to the south of Mexico and stayed there until her husband was accepted as graduate student at Louisiana State University. In November, 1995, she delivered a baby boy, Jose Gilberto, and at the same time, enrolled in the Graduate Program of the School of Forestry, Wildlife, and Fisheries at Louisiana State University, where she is currently a candidate for the degree of Doctor of Philosophy in Wildlife and Fisheries Science.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Carmen G. Paniagua-Chavez

Major Field: Wildlife and Fisheries Science

Title of Dissertation: Cryopreservation of Gametes and Larvae of the Eastern Oyster Crassostrea Virginica

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Robert P. Romaire

Date of Examination:

October 6, 1999